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THE PROCESS OF AMINO ACID FORMATION FROM SUGARS IN ASPERGILLUS NIGER [1]

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INTRODUCTION

A great deal of work has been done on the carbon requirements of the fungi (*13*),[3] and many of their metabolic products have been identified (*10*). Notwithstanding the vast quantity of data accumulated, little is known concerning the fundamentals of the carbon nutrition of these fungi. Though much has been learned regarding the respiratory and fermentative degradation of the sugars, the manner of their employment for synthesis of the protein that is necessary for growth is quite unknown.

The distinction made between the respiratory and fermentative fragmentation of the sugars, on the one hand, and the synthesis of protein for growth, on the other, is considered to be of great importance. *Aspergillus niger* Van Tiegh., for example, can be grown in such a manner that little increment in weight takes place and about 90 percent of the sugar is converted to citric acid. The writer (*16*), on the other hand, has frequently obtained growth equivalent to 52 percent of the sugar supplied. It is evident, therefore, that the relative quantities of carbon assimilate used in these phases of metabolism vary in an extreme manner with environmental conditions.

Observations on the growth of *Aspergillus niger* with glycerol furnished the incentive for the investigations here described. The possibility of improved results with the trace elements by the substitution of glycerol for sucrose had been noted (*15*). It was found, however, that yields with different samples of glycerol ranged from 10 to 400 mg. Though it was found possible to increase yields almost to maximum by the addition of traces of various organic substances, the effective materials were not considered such as were likely to be present as impurities. Moreover, the results on trace elements were rendered poorer, if anything, through the use of such organic compounds, as compared to the high yields obtained with certain samples of reagent glycerol. It seemed advisable, therefore, to make a general study of the carbon assimilation of the fungus in order to arrive at some indication of the type of substances that might be responsible for the observed differences.

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[2] Acknowledgment is made of the kindness of Prof. Claude S. Hudson, of the U. S. Public Health Service, and of Dr. D. Breese Jones, of the Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, in reviewing this manuscript for the writer before its submittal for publication.

[3] Italic numbers in parentheses refer to Literature Cited, p. 633.

Unfortunately, an adequate study of carbon assimilation required the use of many compounds that were unobtainable or obtainable only at great expense because of the small commercial demand. In most instances no one could be found to undertake the preparation of the small quantities needed, either because of the expense involved or because methods of preparation were unknown. It was therefore only through the generosity of investigators in the chemistry of the sugars that the present investigations were made possible. Many gave freely of their own supplies, which were frequently very limited.

Despite such collaboration, it was impossible to carry the work beyond a preliminary stage. Nevertheless, it is believed that enough has been accomplished to indicate clearly the probable course of carbon assimilation involved in the formation of amino acids by the fungus. However, circumstances have required the termination of the studies, and it is doubtful whether they can be resumed.

METHODS AND MATERIALS

The cultural methods employed were similar to those previously described (*14*). *Aspergillus niger* was grown in 200-cc. pyrex Erlenmeyer flasks in all work not involving a direct study of trace elements. Otherwise all laboratory ware used was of transparent fused quartz. Water used in the preparation of nutrient solutions was distilled through a metal still and then through a quartz still.

The standard nutrient solution was prepared by dissolving in a liter of water 50 gm. of sucrose, 1.90 gm. of ammonium nitrate, 0.35 gm. of dipotassium phosphate, and 0.25 gm. of magnesium sulfate (7 H_2O). Iron, zinc, copper, manganese, molybdenum, and gallium were added in concentrations of 0.30, 0.30, 0.075, 0.075, 0.02, and 0.02 mg. per liter, respectively. The fungus was grown on 50-cc. portions of the solution for 4 days at a temperature of 35° C.

The fungus was harvested by filtering the cultures with sintered glass crucibles of No. 3 porosity. The yields were determined after the fungus had been dried overnight at 103° to 105° C.

Substitutions of other carbon compounds [4] for sucrose were effected by omitting the sucrose and adding the compound directly to the culture. It was necessary to check on the acidity that developed in the nutrient solution through the use of compounds other than sucrose and to add sufficient calcium carbonate to bring the solution within a range of pH 5 to 7 when necessary.

[4] The following compounds were obtained through the kindness of the investigators mentioned:
D-Gulose.$\frac{1}{2}CaCl_2.H_2O$, D-α-glucoheptose, D-α-galaheptose, and D-xylonic-γ-lactone, from Dr. Horace S. Isbell, U. S. Bureau of Standards, Washington, D. C.
D-Glyceraldehyde and L-glyceraldehyde, from Prof. E. E. F. Baer and Prof. Hermann O. L. Fischer, Banting Institute, Toronto, Canada.
L-Glucose (preparation of H. O. L. Fischer), from Dr. Harry Rudnick, Banting Institute, Toronto, Canada.
D-Sorbose (preparation of Emil Fischer), from Prof. Hermann O. L. Fischer, Banting Institute, Toronto, Canada.
L-Allose, L-altrose, and L-ribose, from Prof. Fred L. Humoller, Loyola University, School of Medicine, Chicago, Ill.
D-Mannoketoheptose (preparation of F. B. La Forge), from Dr. Elias Yanovsky, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Washington, D. C.
Kojic acid, calcium 2-keto-D-gluconate, calcium 5-keto-D-gluconate, calcium *d*-lactate, and calcium D-gluconate, from Dr. G. E. Ward, Northern Regional Research Laboratory, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Peoria, Ill.
Acetone-D-threose and acetone-D-xyloketose, from Prof. T. Reichstein, Pharmazeutische Anstalt der Universitat, Basel, Switzerland.
L-Tagatose and L-galactose, from Prof. Koichi Iwadare, Chemical Institute, Imperial University, Tokyo, Japan.
D-Ribonic acid lactone and D-arabonic acid, from Dr. Joseph Rosin, Merck & Co., Rahway, N. J.
L-Xylose, from Dr. Hardy W. Larson, Biochemical Laboratory, Metropolitan Life Insurance Co., New York, N. Y.

CARBON UTILIZATION FACTOR

Before attempting to estimate the assimilability of carbon compounds, an experiment was carried out with sucrose (table 1) to ascertain the effect of concentration upon the unit it was planned to use as a basis for comparison. This unit, or "carbon utilization factor," as it will be called, is the value obtained by dividing the yield in grams by the number of grams of carbon present in the substrate; i. e., it is the yield in grams (weighed after drying at 103°–105° C.) per gram of carbon supply.

The carbon utilization factor was found to vary somewhat with the concentration at which the sucrose was employed. It would seem that respiratory and fermentative reactions tended to be emphasized at low concentrations, since values for grams of yield per gram of carbon decreased somewhat. A maximum was reached at concentrations ranging from 50 to 60 percent of optimum. The value again decreased at still higher concentrations because of a presumed greater and greater proportion of unutilized sucrose.

TABLE 1.—*Assimilation of sucrose by Aspergillus niger during 4 days of growth at 35° C.*

Sucrose per culture	Carbon per culture	Yield of fungus	Yield per gram of carbon supply	Relative yield per gram of carbon supply	Sporulation [1]
Grams	*Grams*	*Grams*	*Grams*		
0.075	0.0316	0.0333	1.054	0.898	4
.150	.0632	.0541	[2](.856)	[2](.729)	6
.250	.1053	.1132	1.075	.916	8
.500	.2105	.2333	1.108	.944	8
.750	.3158	.3660	1.159	.987	8
1.000	.4210	.4866	1.156	.985	8
1.250	.5263	.6102	1.159	.987	8
1.500	.6315	.7419	[3] 1.174	1.000	8
1.750	.7368	.8542	1.159	.987	8
2.000	.8420	.9427	1.120	.954	8
2.250	.9473	1.0063	1.062	.905	8
2.500	1.0525	1.0113	.961	.819	8

[1] Rated from 0 (sterile) to 10 (completely covered with spores).
[2] Yield inconsistent with the other values.
[3] The carbon utilization factor of sucrose is therefore equivalent to 1.17.

For accurate comparison of the assimilability of carbon compounds, it was necessary to take the concentration factor into consideration. This was accomplished by dividing the carbon utilization factor for any compound by the relative yield per gram of carbon obtained with sucrose at an equivalent carbon concentration. The term "carbon utilization factor," as employed in this paper, will refer always to such values as have been so corrected for concentration. It was found convenient to plot the relative yields per gram of carbon obtained with sucrose against carbon supply, and thus obtain the intermediate values by inspection.

ASSIMILABILITY OF CARBON COMPOUNDS

Growth and sporulation of the fungus were quite poor with the majority of one-, two-, and three-carbon chain compounds investi gated (table 2). Of the two-carbon compounds tried, glycolic and glyoxylic acid gave best results as nutrients. Nielsen and Hartelius

(*8*) have suggested that these, together with glyceric acid, may function with *Aspergillus* as accessory growth factors. Of the three-carbon compounds, dihydroxyacetone and glyceric acid showed the highest carbon utilization factors. The D– form of glyceraldehyde gave better results than did the L– form. Absence of growth on pyruvic acid had not been anticipated. Lack of growth with the acetal of glycolaldehyde does not necessarily imply that glycolaldehyde would not support growth. The uniform absence of growth with the esters that were investigated may indicate that the strain of fungus used had a low hydrolytic capacity.

TABLE 2.—*Assimilation of organic carbon compounds by Aspergillus niger during 4 days of growth at 35° C.*

Source of carbon supply		Yield of fungus	Carbon utilization factor	Sporulation [1]
Compound	Quantity per culture			
	Grams	*Gram*		
ONE- AND TWO-CARBON COMPOUNDS				
Acetaldehyde	1.0	0.0021	0.0	1, br
Ethylene glycol	1.0	0	0	0
Glycolic acid	1.0	.0136	.04	0
Glyoxylic acid	1.0	.0358	.11	1
Oxalic acid	1.0	0	0	0
Glyoxal, polymerized	1.0	0	0	0
Calcium formate	1.0	0	0	0
Ethyl alcohol	1.0	0	0	0
Calcium acetate	1.0	.0033	.01	0
Glycolaldehyde diethylacetal	1.0	0	0	0
THREE-CARBON COMPOUNDS				
D-Glyceraldehyde	2.5	0.0939	0.11	1
L-Glyceraldehyde	.9433	.0284	.08	6
Dihydroxyacetone	.6452	.0828	.33	8
Glycidol	1.0	0	0	0
Propylene glycol	1.0	0	0	0
Pyruvic acid	1.0	0	0	0
Trimethylene glycol	1.0	.0006	0	0
Glyceric acid	.5	.0415	.25	2
Glycerol	2.5	.0087	.01	1
Allyl alcohol	1.0	0	0	0
Malonic acid	1.0	.0041	.01	1
Acrolein	1.0	0	0	0
Sodium acrylate	1.0	0	0	0
Barium mesoxalate	1.0	0	0	0
Sodium glycerophosphate	1.0	0	0	0
Calcium *d*-lactate	1.0	.0190	.08	2
Propionaldehyde	1.0	0	0	0
Propylene oxide	1.0	[2] +	0	0
FOUR-CARBON COMPOUNDS				
D-Threose [3]	0.25	0.0011	0.01	0
1, 3-Butylene glycol	1.0	0	0	0
2, 3-Butylene glycol	1.0	0	0	0
Succinic acid	1.0	0	0	0
dl-Tartaric acid	1.0	.1081	.39	10
Mesotartaric acid	1.0	.0058	.02	1
l-Malic acid	1.0	.0915	.26	10
dl-Malic acid	1.0	.0121	.03	2
Fumaric acid	1.0	.0834	.20	10
d-Tartaric acid	1.0	.0968	.31	10
Ethyl oxalacetate (sodium salt)	1.0	0	0	0
Ethyl acetoacetate	1.0	0	0	0
Sodium dihydroxytartrate	.5	0	0	0

See footnotes at end of table.

TABLE 2.—*Assimilation of organic carbon compounds by Aspergillus niger during 4 days of growth at 35° C.*—Continued

FIVE-CARBON COMPOUNDS

Source of carbon supply: Compound	Quantity per culture	Yield of fungus	Carbon utilization factor	Sporulation [1]
	Grams	*Gram*		
D-Lyxose	1.0	0.0002	0	0
D-Xylose	2.0	.8602	1.09	10
L-Xylose	.5	.0062	.03	1
D-Arabinose	2.0	0	0	0
L-Arabinose	2.0	.2051	.27	6
D-Ribose	.25	+	0	0
L-Ribose	.25	.0052	.05	0
D-Xyloketose [4]	.25	.0005	.01	0
Glutaric acid	1.0	.0043	.01	0
Levulinic acid	1.0	.0040	.01	0
Itaconic acid	.5	.0024	.01	0
Ethyl acetopyruvate	1.0	0	0	0
Furfural	1.0	0	0	0
Ethyl acetone dicarboxylate	1.0	0	0	0
β-Methyl-D-xyloside	1.0	0	0	0
D-Ribonic lactone	.25	.0025	.03	0
D-Arabonic acid (glassy)	.5	+	0	0
D-Xylonic-γ-lactone	.25	.0071	.08	1

SIX-CARBON COMPOUNDS

D-Galactose	2.5	----------	0.07	----------
L-Galactose	.2	+	0	0
D-Gulose [½ $CaCl_2.H_2O$]	.5079	0	0	0
D-Mannose	2.5	----------	1.46	10
D-Glucose	2.5	----------	1.43	10
L-Glucose	.25	0	0	0
L-Altrose	.25	+	0	0
L-Allose	.25	0	0	0
D-Fructose	2.5	----------	1.38	10
L-Sorbose	2.5	----------	1.26	10
D-Sorbose	.0843	+	0	0
L-Tagatose	.10	.0035	.10	0
D-Digitoxose	.988	0	0	0
L-Fucose	.9788	0	0	0
L-Rhamnose (H_2O)	1.0	.1215	.31	4
L-Ascorbic acid	.25	.0069	.08	0
i-Inositol	.5	+	0	0
Calcium 2-keto-D-gluconate	1.0	.2011	.67	0
Calcium 2-keto-D-gluconate (heated)	1.0	.3120	1.04	10
Calcium 5-keto-D-gluconate	1.0	.0252	.08	1
Calcium D-gluconate	1.0	.0317	.10	2
Kojic acid	1.0	.0001	0	0
Mucic acid	1.0	<.1024	<.30	1
α-Methyl-D-glucoside	.5	.0001	0	0
Calcium citrate	1.0	.0019	.01	0
α-Methyl-D-mannoside	.5	0	0	0
Citraconic anhydride	1.0	0	0	0
D-Isoascorbic acid [5]	1.0	.0991	.24	10
Calcium D-glucuronate	1.0	.2062	.62	10
D-Gluconic lactone	1.0	.0058	.01	0

SEVEN-CARBON COMPOUNDS

D-α-Galaheptose	0.5	0	0	0
D-α-Glucoheptose	.5	+	0	0
D-Mannoketoheptose	.5	0.0063	0.03	0
D-Glucoascorbic acid [5]	1.00	.1577	.39	10

[1] Rated from 0 (sterile) to 10 (completely covered with spores). Color other than black is indicated by br (brown).
[2] Plus sign (+) indicates germination.
[3] Acetone-D-threose was used after decomposition by acetic acid (*11*).
[4] Acetone-D-xyloketose was used after decomposition by acetic acid (*11*).
[5] Substrate became alkaline.

Attention is called to the fact that each oxidation product of glycerol (glyceraldehyde, dihydroxyacetone, and glyceric acid) investigated gave a much better yield than did glycerol. It is evident, therefore, that partial oxidation of reagent glycerol may be a factor in the greater yields often obtained. This cannot be the entire explanation, however, since such substances would be apt to be present only in small concentrations. The acceleration in growth reported to take place on addition of sodium iron chlorophyllin and hemoglobin (*15*) may be associated with an increased oxidative capacity. Increases in yield by amino acids (*14*, *15*) have been shown to be due to the use of a poor carbon source. These would seem to be instances of empirical results whose significance could not be conjectured prior to systematic study.

Responses to four-carbon chain compounds (table 2) were on the whole no better than to one-, two-, and three-carbon derivatives. Superior results were obtained with *dl*-tartaric acid, *d*-tartaric acid, *l*-malic acid, and fumaric acid. Since *dl*-tartaric acid has the configuration of LD-threose, and *d*-tartaric acid that of L-threose, it would seem that the D- form of threose should prove more assimilable than the L- form. D-Threose, however, gave a carbon utilization factor of only 0.01.

Of the seven pentoses investigated, only D-xylose gave a yield approaching that with sucrose (table 2). The reason is not clear for the somewhat better results obtained with L-arabinose and L-ribose than with their D- forms.

The responses given by the fungus with D-xylose, D-lyxose, and D-xyloketose have great significance for an understanding of the process of pentose assimilation. It is well known that the reason assumed for the high and practically equivalent assimilation of D-glucose, D-mannose, and D-fructose is their possession of a common enol; they are epimers. The three pentoses enumerated are also epimers but, unlike the epimeric hexoses just mentioned, give widely different results on assimilation.

Table 2 also gives a summary of the results on assimilability obtained with six-carbon compounds. Only 9 of the 16 aldohexoses could be obtained. Of these, only D-glucose and D-mannose were completely assimilable. Of 5 ketohexoses tried (8 are known), only 2 proved efficacious as sources of carbon; these were D-fructose and L-sorbose. Moderate growth was given by L-rhamnose, D-isoascorbic acid, 2-keto-D-gluconic acid, and D-glucuronic acid.

Oxidation of the aldehyde group in glucose to form gluconic acid had destroyed its nutritive properties. Further oxidation at the 2-carbon atom brought about partial recovery in assimilability, whereas oxidation at the 5-carbon atom did not. Ohle and Berend (*9*) have reported that D-arabinose and D-arabonic acid are formed from 2-keto-D-gluconic acid by heating in solution. These products accounted for less than half the 2-keto-D-gluconic acid present,

however. In view of the unavailability of D-arabinose and D-arabonic acid for growth of *Aspergillus*, it is of interest to note that two periods of heating (20 minutes at 100° C.) increased the assimilability of the 2-keto acid considerably (table 2).

Only with D-glucoascorbic acid, of the seven-carbon compounds (table 2), did the fungus show any appreciable growth. This substance and D-isoascorbic acid gave exceptional results in that the substrates became strongly alkaline through growth of the fungus. L-Ascorbic acid had the lowest carbon utilization factor; D-isoascorbic acid gave a higher value, and D-glucoascorbic acid a still higher value (table 2). In other words, the D- configuration was superior to the L- configuration. The greater usefulness of D-glucoascorbic acid is perhaps due to its partial decomposition at the 1, 2 double bond to form D-glucose or D-mannitol.

CHEMICAL STRUCTURE AND ASSIMILABILITY OF MONOSES

It seems clear from the data of table 2 that the rate of carbon utilization by *Aspergillus* was quite poor with all compounds having less than a five-carbon atom chain. Of the five-carbon compounds, only D-xylose approached maximum efficacy in assimilation. Maximum values for utilization of six-carbon compounds were given by D-glucose, D-mannose, D-fructose, and L-sorbose. That is, only one pentose and four hexoses were found to possess full carbon assimilability.

The basis for the situation just described is attributed to the necessity for preliminary decomposition and condensation reactions when compounds with less than a five-carbon chain are employed. This interpretation is increased in probability by the greater degree of growth given by known degradation products of the sugars. These are dihydroxyacetone, glyceraldehyde, glyceric acid, glycolic acid, and glyoxylic acid. These decomposition reactions probably are reversible to some degree or at least are better fitted for necessary preliminary condensations.

A general idea of the configurations (*5*) and yields of the aldoses and ketoses can be obtained from the following schematic arrangement. The positions of the hydroxyl groups in only the D- aldoses are given, since those of the L- forms can be obtained by substituting minus signs for plus signs, and vice versa. The zeros in the ketose configurational notation represent the keto (=CO) group.

(1) HCO
(2) HCOH +
(3) HOCH −
(4) HCOH +
(5) HCOH +
(6) H_2COH
D–Glucose
Lyxose
D = 0.00; L = ?
Threose
D = −0.01; L = ?
Glyceraldehyde
D = +0.11; L = 0.08
ALDOSES
Erythrose
D = ?; L = ?
L–Glucose
Talose
D = ?; L = ?
Galactose
D = 0.07; L = 0.00
Idose
D = ?; L = ?
Xylose
D = 1.09; L = 0.03
Gulose
D = 0.00; L = ?
Mannose
D = 1.46; L = ?
Arabinose
D = 0.00; L = 0.27
Glucose
D = 1.43; L = 0.00
Altrose
D = ?; L = 0.00
Ribose
D = 0.00; L = 0.05
Allose
D = ?; L = 0.00
(1) H_2COH
(2) HOCH −
(3) HOCH −
(4) HCOH +
(5) HCOH +
(6) H_2COH
D–Fructose
L–Psicose
(?)
L–Arabinoketose
(?)
L–Fructose
(?)
L–Erythrulose
(?)
Dihydroxyacetone
(0.33)
KETOSES
L–Sorbose
(1.26)
L–Xyloketose
(?)
L–Tagatose
(0.10)
D–Fructose
(1.38)
D–Arabinoketose
(?)
D–Psicose
(?)
D–Erythrulose
(?)
D–Sorbose
(0.00)
D–Xyloketose
(0.01)
D–Tagatose
(?)
(1) H_2COH
(2) CO
(3) HCOH +
(4) HOCH −
(5) HOCH −
(6) H_2COH
L–Fructose

A check of structure against yield in the above scheme will disclose that every one of the sugars capable of giving maximum growth has a minimum chain of five carbon atoms and an L-3-carbon atom and a D-4-carbon atom. Monoses deficient in these particulars failed to sustain the maximum rate of growth. These are not the only requirements, since D-lyxose and D-xyloketose have these configurational prerequisites but cannot give maximum growth. The same situation probably exists with L-idose and L-gulose, the epimers of L-sorbose. Monoses having the reverse configuration, as L-gulose in comparison with D-glucose, and L-xylose in comparison with D-xylose, are apparently unable to support growth. The fungus was deficient, therefore, in the ability to transform such compounds to assimilable forms by simultaneous reduction and oxidation of opposite terminal carbon atoms. This process with D-glucose might account for the formation of L-ascorbic acid and L-sorbose in green plants.

These statements may be readily verified through examination of the structural formulas and carbon utilization factors in the following arrangement of monoses. All pentoses and hexoses in which the 3- and 4-carbon atoms have opposite configurations have been included and are indicated by asterisks. Each group of three consists of epimers that are readily convertible into one another by means of a common enol. This relation is indicated by arrows. Response of *Aspergillus* to D-glucose, D-fructose, and D-mannose is similar to that of yeast and would seem to confirm the interpretation that the enol common to these three sugars participates in assimilation. That this assumption is incorrect should prove clear when L-idose and L-gulose become available. *Aspergillus* will probably be found unable to employ these unnatural or synthetic sugars. Gortner (*2*) points out that though some yeasts can slowly ferment D-galactose, they cannot attack D-tagatose and D-talose, its epimers.

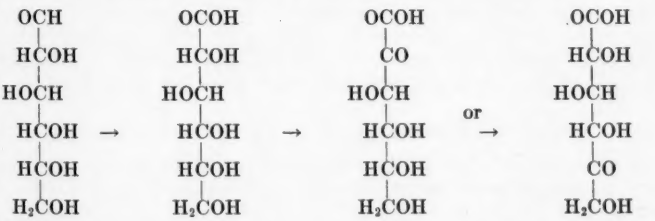
HCO		H_2COH		HCO	(1)	HCO		H_2COH		HCO	
|		|		|	|		|		|	|	
HCOH		CO		HOCH	(2)	HOCH		CO		HCOH	
|		|		|	|		|		|	|	
HOCH		HOCH		HOCH	(3*)	HOCH		HOCH		HOCH	
|	↑↓	|	↑↓	|	|	|	↑↓	|	↑↓	|	|	
HCOH		HCOH		HCOH	(4*)	HCOH		HCOH		HCOH	
|		|		|	|		|		|	|	
HCOH		HCOH		HCOH	(5)	HOCH		HOCH		HOCH	
|		|		|	|		|		|	|	
H_2COH		H_2COH		H_2COH	(6)	H_2COH		H_2COH		H_2COH	
D-Glucose		D-Fructose		D-Mannose		L-Gulose		L-Sorbose		L-Idose	
(1.43)		(1.38)		(1.46)		(?)		(1.26)		(?)	
HCO		H_2COH		HCO	(1)	HCO		H_2COH		HCO	
|		|		|	|		|		|	|	
HOCH		CO		HCOH	(2)	HCOH		CO		HOCH	
|		|		|	|		|		|	|	
HCOH		HCOH		HCOH	(3*)	HCOH		HCOH		HCOH	
|	↑↓	|	↑↓	|	|	|	↑↓	|	↑↓	|	|	
HOCH		HOCH		HOCH	(4*)	HOCH		HOCH		HOCH	
|		|		|	|		|		|	|	
HOCH		HOCH		HOCH	(5)	HCOH		HCOH		HCOH	
|		|		|	|		|		|	|	
H_2COH		H_2COH		H_2COH	(6)	H_2COH		H_2COH		H_2COH	
L-Glucose		L-Fructose		L-Mannose		D-Gulose		D-Sorbose		D-Idose	
(0.00)		(?)		(?)		(0.00)		(0.00)		(?)	
HCO		H_2COH		HCO	(1)	HCO		H_2COH		HCO	
|		|		|	|		|		|	|	
HCOH		CO		HOCH	(2)	HOCH		CO		HCOH	
|		|		|	|		|		|	|	
HOCH		HOCH		HOCH	(3*)	HCOH		HCOH		HCOH	
|	↑↓	|	↑↓	|	|	|	↑↓	|	↑↓	|	|	
HCOH		HCOH		HCOH	(4*)	HOCH		HOCH		HOCH	
|		|		|	|		|		|	|	
H_2COH		H_2COH		H_2COH	(5)	H_2COH		H_2COH		H_2COH	
D-Xylose		D-Xyloketose		D-Lyxose		L-Xylose		L-Xyloketose		L-Lyxose	
(1.09)		(0.01)		(0.00)		(0.03)		(?)		(?)	

The behavior of D-xylose and its epimers affords a similar proof that enolization is not concerned in assimilation. D-xylose gave excellent growth; D-xyloketose permitted germination; and D-lyxose gave no germination.

The differences indicated in behavior of epimers are presumably associated with requirements for initial attack. The chains shown as straight, for convenience, do not exactly conform to that structure, but even on the basis of furanose and pyranose rings, no explanation of these differences in behavior is obvious. The pyranose structures of D-glucose and its epimers are identical with those of D-xylose and its epimers, except that they contain a "—CHnOH—" instead of a_2 "—H" on their fifth carbon atoms (*4*). These modifications result in very different effects.

The use of *Aspergillus* instead of yeast should prove of great aid in the study of these phenomena, since the number of epimeric groups, and therefore the number of derivatives suitable for study, is thereby greatly increased. Yeast, as a matter of fact, cannot be used, since the only group of epimers it can attack shows practically no differences in behavior of the individual sugars. Studies with compounds having an L–3- and L–4-carbon atom that are unassimilable (D-xyloketose, D-lyxose) or are readily transformed to such theoretically (L-gulose, L-idose) appear desirable for an understanding of the requirements for initial attack.

Instances showing the importance of a 1-, 2-, 5-, or 6-carbon atom in attack and assimilation of the hexoses have been found. Helferich and Bigelow (*3*) stated that yeast is unable to ferment 5-keto-D-glucose, whereas Micheel and Horn (*6*) found that 5-keto-D-fructose was only about half as effective as glucose. Fischer (*1*) found that yeast cannot ferment D-glucosone (2-keto-D-glucose). With *Aspergillus* the introduction of an amino group at the 2-carbon atom (D-glucosamine) brings the carbon utilization factor down to 0.03. Moreover, as shown in the following arrangement, the oxidation of D-glucose to D-gluconic acid renders the sugar practically unassimilable, though with further oxidation to 2-keto-D-gluconic acid it becomes about half as effective as D-glucose. Oxidation of the 5-carbon atom of D-gluconic acid does not increase assimilability.

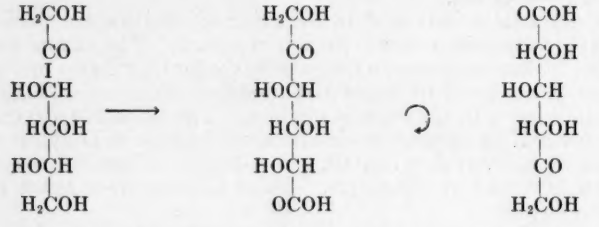


D-Glucose (1.43)	D-Gluconic acid (0.10)	2-Keto-D-gluconic acid (0.67)	5-Keto-D-gluconic acid (0.08)

While oxidation of D-glucose at the 1-carbon atom to D-gluconic acid renders it practically unassimilable, oxidation at the 6-carbon atom (to D-glucuronic acid) only halves assimilability (C. U. F.[5]= 0.62). Another instance, in which oxidation at the 6-carbon atom has the opposite effect, is shown by comparing L-sorburonic acid

[5] Carbon utilization factor.

(i. e. 5-keto-D-gluconic acid) and L-sorbose, as shown in the following arrangement. It is necessary to rotate the structural formula of the former through 180° in the plane of the page.



L-Sorbose (1.26) | L-Sorburonic acid (0.08) | 5-Keto-D-gluconic acid (0.08)

The absence of results with a large number of oxidation products of D-glucose makes interpretation of their behavior difficult and somewhat speculative. The important distinction to be kept in mind in this connection is that even minute modifications in structure usually render the sugar completely unassimilable. An alteration in structure that does not do so probably approaches that of the true intermediate.

It would seem likely as a result of these data that α-keto-D-glucuronic acid or a derivative is the intermediate that undergoes decarboxylation. In this connection, investigation should be made of assimilability of the 2- and 5-keto-D-glucosaccharic acids. These have not yet been prepared, however. Tamiya (*17*) found D-glucosaccharic acid (saccharic) 30- to 60-percent assimilable by *Aspergillus oryzae*. Introduction of an α-keto group should have the effect of further increasing assimilability, as occurred with D-gluconic acid.

Assimilation of hexose, would seem on this basis to comprise a highly complicated series of enzymatic reactions. These include oxidation to an α-keto acid and decarboxylation. Whether the other terminal carbon atom is oxidized to carboxyl during the first stage or whether this oxidation takes place in the pentose derivative that is formed remains to be determined. Its presence in the amino acids definitely indicates its formation, however. Moreover, the constitution of the amino acids requires removal of hydroxyl from pentose or hexose in their formation, accomplished possibly through dehydration and reduction.

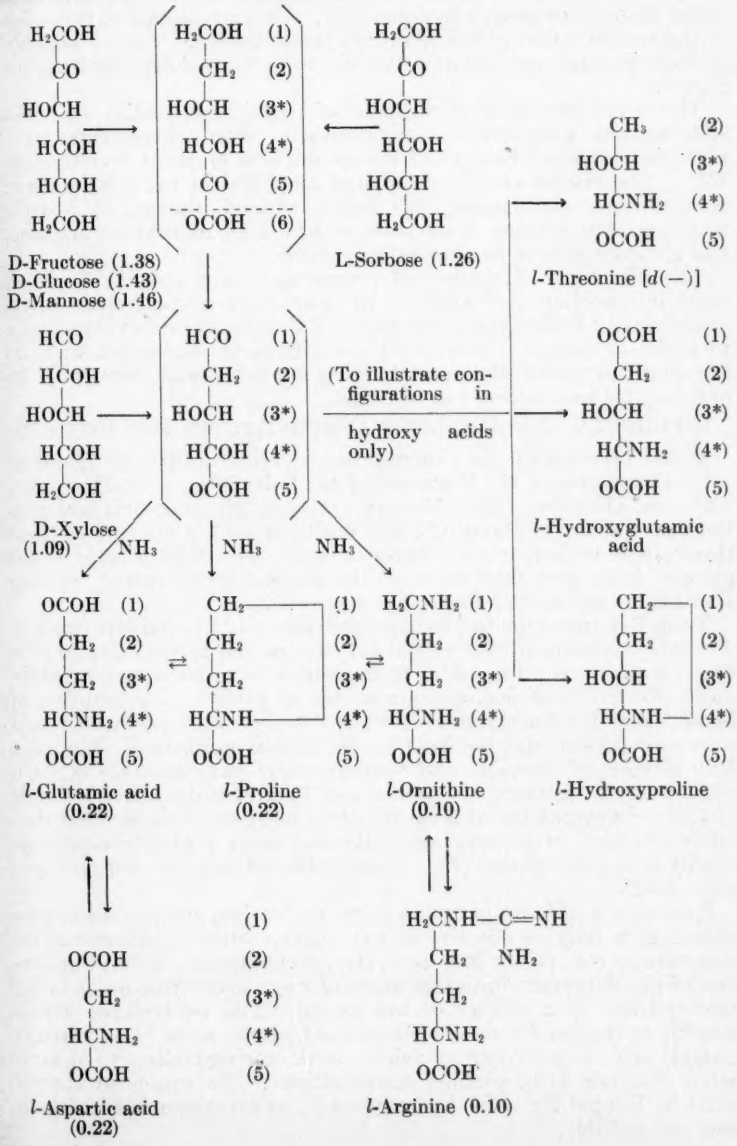
CONVERSION OF SUGARS TO AMINO ACIDS

Any hypothesis concerning the transformation in *Aspergillus* of sugars to amino acids must provide explanations for the following facts:

(1) Use of ammonia in formation of the amino group in amino acids (*14*).
(2) Direct synthesis of primary amino acids: Proline, glutamic acid, ornithine (*16*).
(3) Presence of levo amino groups alpha to carboxyl in amino acids.
(4) Presence of beta hydroxyl groups in all hydroxyamino acids, but no gamma or delta group.
(5) Presence of dextro hydroxyl groups beta to carboxyl in amino acids.
(6) Relative unassimilability of compounds with less than a five-carbon atom chain.
(7) Necessity for L-3- and D-4-carbon atoms in assimilable sugars.

(8) Unassimilability of certain sugars by certain organisms.
(9) Unassimilability of D-xyloketose, D-lyxose, and probably L-idose and L-gulose.
(10) Relatively better assimilability of glyceraldehyde, glyceric acid, dihydroxyacetone, glycolic acid, and glyoxylic acid.

A series of chemical reactions meeting the requirements named is as follows:



A detailed discussion of each point is unnecessary. It should be understood, however, that it is intended only to indicate the general paths followed in these transformations. No evidence was obtained for participation of the intermediates shown nor for their number. A degree of certainty exists only as concerns the initial and end products.

Threonine, serine, hydroxyglutamic acid, and hydroxyvaline are beta hydroxy acids of natural occurrence, whereas hydroxyproline is either the beta or gamma hydroxy acid. Only in the case of threonine is the configuration of the hydroxyl group known. Except possibly hydroxyglutamic acid, none is thought to be formed directly from the sugars.

The direct formation of glutamic acid, ornithine, proline, and possibly aspartic acid from a sugar derivative with a five-carbon atom chain is postulated because of the results of a previous investigation (*16*). The results are discussed and amplified in the next section. Schoenheimer and Ratner (*12*) found, through the use of isotopic nitrogen, that in mice conversion of ornithine to proline, arginine, and glutamic acid is an automatic reaction.

The dihydroxy-aldehyde-acid represented as a possible 5-carbon atom intermediate compound is the α-hydroxy acid-aldehyde corresponding to β-hydroxyglutamic acid. This amino acid has been found to occur in nature. Because of the difficulties associated with its isolation, very little of this material is available, and none could be obtained for use in these experiments.

EFFICIENCY OF ANABOLITES AS SUBSTITUTES FOR MONOSES

It has been shown that sucrose has a carbon utilization factor of 1.17; D-mannose, 1.46; D-glucose, 1.43; D-fructose, 1.38; D-sorbose, 1.26; and D-xylose, 1.09. Moreover, though glutamic acid and proline gave factors of about 0.22 and ornithine 0.10, a mixture of these three amino acids reached a factor of 0.83. This is 58 percent of the glucose value and may indicate the proportion of carbon passing through an amino acid stage during growth.

Table 3 contains the results of miscellaneous additional experiments. Though pyruvic acid by itself did not even permit germination to take place, it appeared, when added to an amino acid mixture, to be slightly more effective than sucrose as a source of carbon. Substitution of *l*-malic acid for *l*-aspartic acid when *l*-proline and *d*-glutamic acid were also present did not lead to diminished utilization. Use of a 5 : 9 mixture of *l*-proline and *l*-aspartic acid gave a factor of 0.63, while a similar mixture of *l*-proline and *l*-malic acid reached a factor of 1.12. In evaluation of these results it must be borne in mind that interconversion of aspartic and glutamic acids probably occurs as readily as in green plants (*7*). Deamination of aspartic acid produces malic acid.

Evidently a mixture of amino acids and carbon compounds is possible that is fully as effective as any sugar. Since requirements for this mixture are specific in nature, the probability exists that duplication of the important initial products of sugar decomposition is being accomplished to a greater or less extent. Full equivalence would possibly be reached by use of a mixture of hydroxy acids—*l*-α-hydroxyglutaric acid, *l*-α,δ-dihydroxy-valeric acid—corresponding to deaminated glutamic acid, proline, and ornithine. No source of supply could be located for these hydroxy acids, so experiments with them were not possible.

TABLE 3.—*Carbon utilization factors for mixtures of compounds obtained with Aspergillus niger after 4 days of growth at 35° C.*

Source of carbon		Mixtures of carbon compounds in cultures on which *Aspergillus niger* was grown																			
l-Aspartic acid	gm	0.25		0.25	0.25	0.25	0.25	0.45	0.45	0.45				0.25	0.25	0.25	0.25				0.25
d-Glutamic acid	gm	.25	0.25		.25	.25	.25				0.45	0.45	0.45	.25	.25	.25	.25		0.25	0.25	.25
l-Proline	gm	.25	.25	.25		.25	.25	.25	.25	.25	.25	.25	.25	.25	.25	.25	.25	0.25	.25	.25	.25
d-Arginine hydrochloride	gm					.25			.25			.25									
d-Ornithine dihydrochloride	gm						.25			.25			.25								
dl-Alanine	gm													.25							
l-Leucine	gm														.25						
l-Hydroxyproline	gm															.25					
l-Malic acid	gm																	.45	.25		
Fumaric acid	gm																			.25	
Pyruvic acid	gm																.25				
Sucrose	gm																				.25
Carbon utilization factor (sucrose=1.17)		0.66	0.45	0.49	0.17	0.79	0.82	0.63	0.81	0.82	0.77	0.74	0.83	0.46	0.70	0.40	0.89	1.12	0.83	0.83	0.80

TRACE-ELEMENT REQUIREMENTS WITH THE MONOSES

A résumé of the trace-element requirements of the fungus with the monoses is given in table 4. Nutrient solutions purified by treatment with calcium carbonate were employed in making these tests (*14*). The data for the different sugars are quite uniform, with one exception: D-xylose gave an exceptionally high yield in cultures from which the iron had been removed. The reason for this response was not investigated. The data would definitely refute the claim that has been made in the past that zinc is necessary only for the utilization of sucrose.

DISCUSSION

Investigations into the biological conversion by plants of sugar to amino acids have been inconclusive in the past, it is believed, because of the choice of unsuitable organisms for study. Green plants furnish unfavorable material because the quantity and nature of the sugar to be employed cannot be controlled in a normal environment. Bacteria are unsuitable because of very low yields as contrasted to quantity of dissimilation products. Fungi, however, are uniquely fitted for such studies, if, like *Aspergillus*, they are of unspecialized nutrition.

The use of cultivated yeasts, to the practical exclusion of other fungi, has been an unfortunate choice. Not only are these organisms practically confined to the use of D-glucose and its epimers, but they are also characterized by a great predominance of fermentative reactions. It is possible in this connection that use of D-galactose by "trained" yeasts depends on its complete break-down and the recombination of the fragments. *Aspergillus niger*, however, assimilates D-xylose and L-sorbose in addition, and can convert a large proportion of these sugars to cellular material under conditions where fermentation is practically suppressed. *Aspergillus*, moreover, would seem to differ less from green plants than the yeasts, since D-xylose (wood sugar) and L-sorbose (L-ascorbic acid configuration) are also participants in the metabolism of the green plants.

Assimilation of hexoses would seem to depend primarily on the presence of an L-3-carbon and a D-4-carbon configuration. Only hexoses meeting this requirement support growth since they meet the configurational requirements of the amino acids. Initial attack may occur at the 1- or 6-carbon atom, a derivative of an α-keto acid probably being formed. Decarboxylation results in the formation of a compound having a chain of five carbon atoms. Probably L-gulose, like L-xylose, cannot be modified by the fungus to meet its requirements, though simultaneous oxidation and reduction at opposite ends of the carbon chain would accomplish this change.

Oxidation and decarboxylation reactions probably account for specificity in initial attack of the hexoses. *Aspergillus* utilizes all hexoses having a satisfactory 3-,4-carbon atom configuration, except probably L-gulose and L-idose, whereas yeasts cannot attack these or L-sorbose. This distinction in capacity between these two kinds of fungi would indicate the probability that break-down of D-glucose and L-sorbose is dependent upon different enzymatic systems. The differences shown by D-xylose, D-lyxose, and D-xyloketose with *Aspergillus* would also indicate a very delicate adjustment in enzymatic attack. The only distinction between these compounds is that they possess a D-2-carbon, an L-2-carbon, and a 2-keto-carbon

TABLE 4.—*A comparison of trace-element requirements of Aspergillus niger with different sugars when grown at 35° C. for 4 days in solutions purified with calcium carbonate*

Element omitted	D-Glucose					D-Fructose				L-Sorbose						D-Mannose					D-Xylose				
	Yield per 2.5 gm. D-glucose	Relative yield	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. D-fructose	Relative yield	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. L-sorbose	Relative yield	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. D-mannose	Relative yield	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. D-xylose	Relative yield	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Milligrams	*Percent*	*pH*			*Milligrams*	*Percent*	*pH*			*Milligrams*	*Percent*	*pH*			*Milligrams*	*Percent*	*pH*			*Milligrams*	*Percent*	*pH*		
None	1,152.9	100.00	3.36	3	10	1,131.3	100.00	4.00	E	10	1,059.8	100.00	2.57	0	8	1,211.2	100.00	2.99	2	10	1,053.0	100.00	5.38	E	10
Fe	4.5	0.39	3.22	0	0	17.9	1.58	3.68	0	2	21.3	2.01	3.36	0	4	13.5	1.11	3.63	0	1	1,005.3	95.46	2.30	0	6
Zn	21.6	1.88	2.87	0	1	65.6	5.80	2.59	0	8	25.8	2.43	3.50	0	4	21.2	1.75	3.28	0	3	39.3	3.73	2.78	0	2
Cu	860.0	74.60	2.48	2	2, w	940.5	83.14	3.08	1	4,w	1,049.8	99.05	2.58	0	8	899.5	74.27	2.73	0	4,y	1,021.0	96.95	3.86	0	10,t
Mn	685.4	59.45	1.62	*5(3)	0	1,016.9	89.88	1.62	*3	4	664.1	62.66	1.67	*5(1)	1	774.5	63.95	1.57	*5(1)	4	1,057.3	100.41	4.49	E	10
Mo	1,137.2	98.65	2.27	3	6	996.5	88.08	3.17	0	10	995.7	93.95	2.23	1	6	1,106.4	91.34	2.29	0	6	1,018.1	96.68	1.77	*5(5)	4
Ga	1,119.7	97.13	3.42	3	10	1,130.4	99.92	3.86	0	10	1,039.4	98.08	2.54	0	8	1,181.4	97.54	3.49	2	10	1,051.6	99.86	4.48	E	10
Max.[3]	1,197.2					1,164.1					1,060.8					1,228.5					1,068.4				
C. U.[4]		47.89					46.56					42.43					49.14					42.74			
pH [5]			7.17					6.97					6.25					7.12					6.68		

[1] Starch is rated on a scale of 0 (absent) to 5 (profuse), an astreisk (*) indicating immediate formation of a blue color on addition of N/20 iodine solution. Values in parentheses represent comparable data for the substrates. E=erythrodextrin.
[2] Rated from 0 (sterile) to 10 (completely covered with spores). When spores were not black, color is indicated by t (tan), y (yellow), and w (white).
[3] Maximum individual yield.
[4] Coefficient of utilization, or yield per 100 gm. of sucrose.
[5] Initial acidity of the nutrient solution.

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atom, respectively. It seems possible that enzymatic reduction at the 2-carbon atom is involved with the pentoses, though this may be accompanied by oxidation at other points. The D-xylose and the L-sorbose enzyme systems would seem to be absent in the yeasts.

While amino acid configurational requirements for levo-α-amino and dextro-β-hydroxyl groups sharply limit the list of assimilable monoses, a mixture of primary amino acids was almost as effective in maintaining growth. Neither the ornithine, glutamic acid, nor proline comprising this mixture possessed an asymmetric carbon atom (hydroxyl) in the 3 position. The explanation for this seeming contradiction is not obvious in the light of our present knowledge. A simple initial procedure that may prove of aid for investigation of this difference might be based on the requirements for a mixture fully equivalent to the D-glucose.

Absence of vigorous growth on glycerol would seem to depend on an oxidative incapacity of the fungus. Glyceraldehyde, dihydroxyacetone, glyceric acid, glyoxylic acid, and glycolic acid—all probable oxidation products of glycerol—gave better growth than did glycerol. The tartaric acids behaved similarly as compared to D-threose. The condensation mechanism whereby short-chain inactive molecules are combined to form optically active compounds is the opposite of the ordinary process of nutrition. This is true in the preliminary stages at least. There is some evidence that dissimilative break-down of hexose to triose phosphate is reversible. An interesting phase of comparative studies of growth on sucrose and on glycerol concerns itself with possible changes in quantitative requirements for trace elements in the two types.

SUMMARY

Carbon utilization factors (grams of yield per gram of carbon supply) for about 120 compounds, obtained with *Aspergillus niger* Van Tiegh. grown for 4 days at 35° C., were as follows: D-glucose, 1.43; D-mannose, 1.46; D-fructose, 1.38; L-sorbose, 1.26; and D-xylose, 1.09. Other compounds were poor sources of carbon supply. Enolization was not a factor in sugar utilization. All pentoses and hexoses having an L-3-carbon atom and a D-4-carbon atom were assimilable, except the epimers of D-xylose and probably L-sorbose. Preliminary oxidation to α-keto acid derivatives and decarboxylation appeared necessary for assimilation of hexoses.

The hypothesis was proposed that the aldehyde derivative of *l*-α,β-dihydroxy *n*-valeric acid served as precursor for amino acid formation. D-4-carbon of the monoses became *l*-α-amino carbon in the amino acids, and L-3-carbon remained unchanged to become *d*-β-hydroxy in *d*(-)threonine, and possibly in hydroxyglutamic acid and hydroxyproline also. The primary amino acids formed were probably proline, glutamic acid, and ornithine—all α,δ-derivatives of *n*-valeric acid—since mixtures of these attained a carbon utilization factor of 0.83 as compared to 1.17 with sucrose. Evidence was obtained that the carbon utilization factor for amino acid mixtures was increased by substitution of the corresponding hydroxy acids.

Growth requirements for trace elements with assimilable sugars showed little difference, except that cultures on D-xylose from which iron had apparently been removed by treatment with calcium carbonate gave exceptionally high yields. Trace-element requirements with monoses were quite similar to those with sucrose.

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THE RELATION OF ZINC TO SEED PRODUCTION [1]

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INTRODUCTION

The importance of zinc for the complete development of many agricultural plants has been demonstrated by numerous workers in recent years. The subject has gained additional interest from the discovery that certain soils do not supply the amounts of zinc necessary for every crop.

REVIEW OF LITERATURE

The zinc in many noncultivated forest soils, according to Hibbard (*5*),[3] tends to accumulate in zones less than 5 inches below the surface as a result of the deposition of leaves and twigs and the rapid fixation of zinc when liberated by the processes of decay.

Sommer and Lipman (*12*) and Chandler (*3*) presented conclusive evidence that zinc is a necessary element for the growth of a variety of agricultural plants. Chandler's observation that zinc deficiency is responsible for the imperfect development of fruits and nuts of various orchard trees is of especial interest in connection with the subject of this paper. He found that blossoms borne on trees suffering from zinc deficiency failed to set a normal crop of fruit. The yield was therefore defective in quantity as well as quality. An application of a small amount of zinc during the winter resulted, however, in the production of healthy foliage and a normal crop of fruit on trees that had formerly manifested symptoms of serious zinc deficiency.

It is intended in this paper to present the results of experiments to determine the relation of zinc to seed production in peas (*Pisum sativum* L.), beans (*Phaseolus vulgaris* L.), and dwarf milo (*Sorghum vulgare* Pers.). The relation of the microelements to seed production is a matter of very great importance, since many agricultural crops are grown almost exclusively for the seed produced. It is obviously difficult to differentiate the effects of an element on the vegetative and reproductive activities of the plant because of the close interrelationship of the two processes and their dependency upon each other. Nevertheless, a beginning has been made, and the results of the experiments may be noted briefly.

The function of zinc in the reproductive processes of plants was first studied in the mold fungi. Although the process of conidial formation in fungi is obviously quite different from reproduction in the angiosperms, it will yet be profitable to notice a few recent studies on the phenomenon.

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[1] Received for publication July 9, 1941.

[2] Valuable assistance in the care of the cultures was rendered by the Work Projects Administration (officia project No. 65-1-05-91, B10) and by W. H. Greenleaf, who also assisted in preparing the data for publication.

[3] Italic numbers in parentheses refer to Literature Cited, p. 643.

Butkewitsch and Orlov (*2*) reported that zinc sulfate accelerated mycelial growth and increased the efficiency of the utilization of sugar by the fungus, although a wide variation in concentration of zinc did not cause marked differences in the growth of the mycelium. Steinberg (*13*) and Gollmick (*4*) found that the presence of a small amount of iron, zinc, copper, and manganese is essential for the normal growth and sporulation of *Aspergillus niger*. Lockwood, Ward, and May (*7*) reported that the addition to the culture medium of 10 mg. per liter of zinc as zinc sulfate increased the weight of mycelium, the consumption of glucose, and the production of dextrolactic acid by *Rhizopus oryzae*. Waksman and Foster (*15*) arrived at somewhat different conclusions. They reported that zinc stimulates growth and respiration but does not affect the accumulation of lactic acid in the culture. Lockwood (*6*) found that spore germination of *R. oryzae* was favored by the presence of zinc, and further reported that in a concentration of 0.01 mg. of zinc per liter of solution, germination and sporulation were accelerated, but the yields of lactic acid were inversely proportional to the concentration of zinc in the culture solution.

Sommer's experiments demonstrated (*11*) that zinc was highly important for the reproductive processes of Windsor beans. The growth of the plants in her experiments was not seriously restricted by the lack of zinc until the flowering stage was reached; the deficiency was then shown by the abscission of leaves and flower buds. The control plants which received zinc grew well, blossomed freely, and set seeds. At maturity, the average dry weight of the bean plants without zinc was 13.3 gm., the weight with zinc 27.0 gm.

In experiments with corn (*Zea mays*) by the present writer and Beck (*9*), it was found that the production of cobs and kernels was relatively more reduced by zinc deficiency than the production of stalks, leaves, and husks. Three consecutive crops of corn were grown to maturity in small tanks of soil, half of which were supplied with nutrients and microelements, except zinc; the other half received the same supplies with the addition of zinc. The effects of zinc were not well defined in the first, but were perfectly evident in the second and third crops. In the third crop the production of cobs and kernels was small.

Bobko and Zerling (*1*) found that the greatest concentration of boron was regularly in the parts of the plant associated with fecundation, and showed that the addition of boron increased the percentage of germination of pollen grains and the length of pollen tubes produced therefrom. Manganese, zinc, iodine, and copper had similar but less obvious effects.

Parker (*8*) applied a zinc sulfate solution prior to the blooming period to grapefruit trees affected with mottle-leaf. He found that, although zinc deficiency had formerly greatly reduced the crop, the application of zinc produced an increase in the quantity and an improvement in the quality of the fruit in the first season after spraying. While there was also an improvement in the vegetative condition of the trees, the improvement in the fruit was so immediate that it could not have been related solely to the development of better foliage. The more severely affected trees in the orchard responded most strikingly to the treatment.

MATERIALS AND METHODS

Experiments on the relation of zinc to seed production were carried out with Dwarf Telephone peas, Davis Dwarf Wax beans, and Dwarf milo. The plants were grown to maturity in nutrient solutions made with extreme care. The composition of the nutrient solutions was that given by Stout and Arnon (*14*), except the concentrations of zinc. As shown in figure 1, one set of cultures received all the microelements except zinc, others received 0.005, 0.02, 0.10, and 0.20 parts per million of zinc. Dwarf milo plants grew so large as to need additional applications of zinc at various intervals. The original amounts added were those stated above, but the applications were repeated three times, giving, therefore, four times the original amount of zinc furnished, which was necessary to keep the plants in a good state of health. All experiments were made in pyrex glass beakers, in a well-lighted greenhouse during the summer months, in Berkeley, Calif.

The amount of zinc in samples of the original seeds was kindly determined by Dr. P. R. Stout, by means of the polarigraph. The peas contained from 8.1 to 8.5 gammas per seed, or an average of 25 p. p. m. of air-dry weight. The milo seeds contained from 0.63 to 0.67 gammas per seed, or, expressed on the basis of air-dry weight, from 20.1 to 21.1 p. p. m. The seeds were washed in redistilled water to remove adhering particles of dust from the surface, and germinated in acid-washed sand. They were later transferred to the special plaster of paris covers utilized to support the seedlings in the beakers. Three plants were usually installed in beakers, each containing 2 liters of the appropriate nutrient solution.

It was necessary to conduct air through the nutrient solution in order to maintain a healthy growth of roots of peas. This was done by blowing a fine stream of air through a sintered glass tube made especially for the purpose. In the nonaerated cultures, the roots of peas became gelatinous and died within about 3 weeks from the time that the experiment was started. The wax beans and milo grew better without aeration than peas, but both responded by better growth when aerated. Since both peas and beans are normally self-pollinated, they served as good test subjects. The dwarf milo, which is supposed to be wind-pollinated, received insufficient pollination to form seeds under the conditions of these experiments and the plants were not as reliable indicators of the effects of zinc as the peas and beans. The weight of the heads of the milo plants was taken, however, since it seemed to be indicative of the processes leading up to seed formation. When each experiment was terminated tops, roots, pods, and seeds were separated and weighed. The seeds that were to be tested for germinating power were dried in the air; the other parts were dried in the oven at temperatures near 100° C.

The value of the several variables are expressed as percentages of the sums of each variable (fig. 1). This method of expression makes it possible to compare the values of the dependent variables on a uniform basis. As a necessary adjunct, the analyses of variance are presented in table 2, without which it would be impossible to estimate the reliability of the results of varying the amounts of zinc furnished the plants.

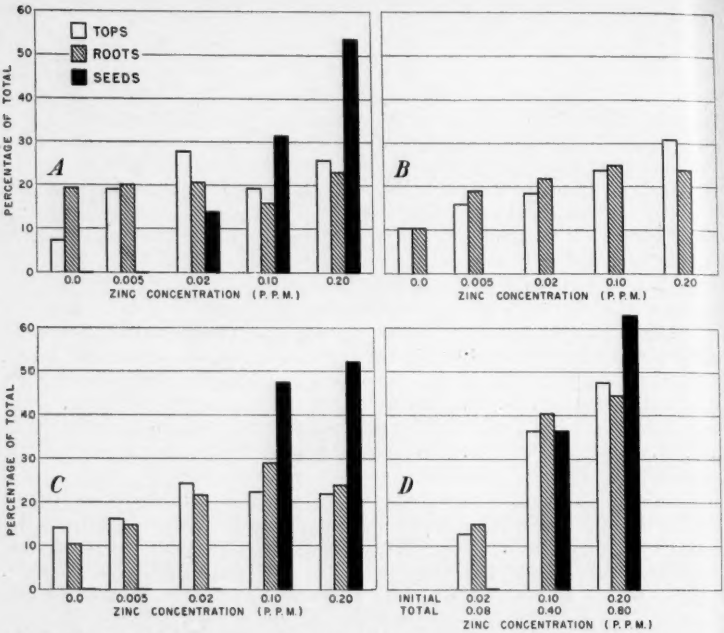

FIGURE 1.—Weight of tops, roots, and seeds of peas, wax beans, and dwarf milo as affected by the quantity of zinc supplied in the nutrient culture: *A*, Dwarf Telephone peas, aerated (experiment 267); *B*, Davis Dwarf Wax beans, aerated and nonaerated combined (experiment 276); *C*, Davis Dwarf Wax beans, aerated (experiment 285); *D*, dwarf milo (experiment 346).

EXPERIMENTAL RESULTS

PEAS

In experiments to determine the effect of zinc on the production of seed, Dwarf Telephone peas were grown in water cultures, some of which were aerated and others unaerated. The cotyledons were removed when the seedlings were transferred to the cultures. In each set of cultures there were 15 beakers containing nutrient solution. The controls contained all microelements except zinc; the other cultures received 0.005, 0.02, 0.10, and 0.20 p. p. m. of zinc.

Seedling peas were installed in the pyrex beakers on May 24 according to the technique outlined above. For a short time they made very little growth, probably because of the removal of the cotyledons, but later they grew better and reached stages of development obviously related to the supply of zinc. Without forced aeration the roots were unable to grow for more than a short time. On June 14 the roots of plants in nonaerated cultures were dead and gelatinous and consequently the tops had made less growth than those of the plants in aerated cultures. All plants, however, were beginning to form flowers on that date. The plants that grew in the aerated cultures were harvested on July 31 and the seeds counted.

The important relations of zinc to the growth of the pea plant (tops and roots) as well as to the seeds are shown in figure 1, A. Increases in the supply of zinc were reflected to a slight degree in the dry weight

of tops, but not at all in the weight of roots when the weight in each culture was expressed as a percent of the combined weight of all cultures. The number of seeds or their weights, on the contrary, show a remarkably close relation to the amounts of zinc supplied (table 1). In the presence of 0.005 p. p. m. of zinc, there was no production of seed, but in 0.02 p. p. m. a significant number of seeds were produced, and at the higher concentrations seed production was materially augmented.

TABLE 1.—*Effect of the quantity of zinc supplied on the average number of seeds produced per culture by Dwarf Telephone peas and Davis Dwarf Wax beans*

Experiment No.[1]	Plants and treatment	Seeds produced when indicated quantity of zinc was supplied (p. p. m.)				
		0	0.005	0.02	0.10	0.20
		Number	*Number*	*Number*	*Number*	*Number*
267	Dwarf Telephone peas (aerated)	0	0	13	24.3	38.0
276	Davis Dwarf Wax beans (aerated)	0	0	3	34.3	28.0
	Davis Dwarf Wax beans (nonaerated)	0	0	2	44.7	27.7
285	Davis Dwarf Wax beans (aerated)	0	0	0	17.3	16.0

[1] In experiments 267 and 276 each figure is the average of 3 cultures of 3 plants each, in experiment 285 of 6 cultures of 3 plants each.

The probabilities were more than 99 to 1 that the differences in the weights of seeds and of number of seeds were real differences (table 2); but differences in the dry weights of roots were not statistically significant.

Further insight into the functions of zinc can be obtained from the following observations on the viability and growth of the seeds produced in this set of cultures. A high percentage germinated from all except the minus-zinc cultures (table 3), in which the single seed that germinated produced only a small depauperate plant. Figure 2

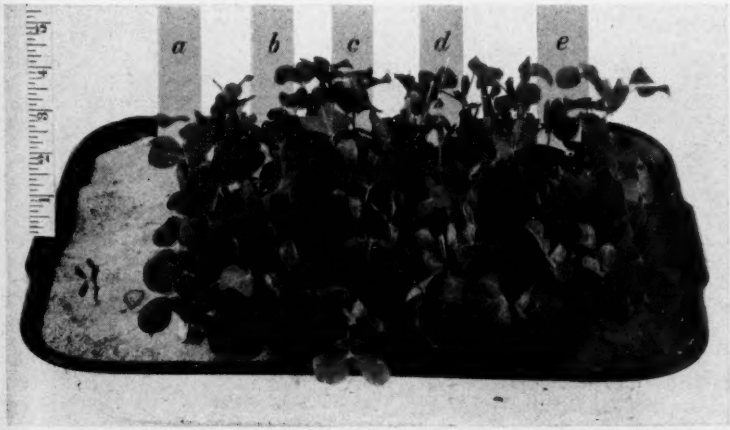

FIGURE 2.—Seedlings produced from seeds of pea plants grown in experiment No. 267 in nutrient cultures containing different quantities of zinc: *a*, Beakers 1–3, parent plants received no zinc; *b*, beakers 4–6, parent plants received 0.005 p. p. m. of zinc; *c*, beakers 7–9, parent plants received 0.02 p. p. m. of zinc; *d*, beakers 10–12, parent plants received 0.1 p. p. m. of zinc; *e*, beakers 13–15, parent plants received 0.2 p. p. m. of zinc. Plants photographed June 20, 1939, 20 days after planting. See table 3.

shows the vigorous seedlings that came from these seeds despite the fact that some of the series received very small amounts of zinc and the seeds produced were not filled out as well as those in the normal plants. The cotyledons of the parent plants had been, as previously stated, removed at the time the plants were installed in the water cultures.

TABLE 2.—*Analysis of variance between treatments for plants that received different quantities of zinc in their nutrient cultures*

Plant, experiment No., and item compared	Degrees of freedom	Mean square between treatments	*F*	Value of *F* required for probabilities of—	
				95:5	99:1 [1]
Peas; experiment 267:					
Dry weight of tops	4	41.79	5.30	3.48	5.99
Dry weight of roots	4	.23	2.96	5.96	
Dry weight of seeds [2]	2	46.70	44.06		10.92
Number of seeds [2]	2	470.11	53.56		10.92
Beans; experiment 276:					
Dry weight of leaves	4	2.96	7.45		4.43
Dry weight of roots	4	.52	4.03	2.87	4.43
Number of pods	4	327.00	18.33		4.43
Number of seeds [3]	1	408.34	3.73	5.32	
Beans; experiment 285:					
Dry weight of leaves	4	9.21	7.61		4.18
Dry weight of roots	4	9.17	19.30		4.18
Dry weight of pods	4	31.60	2,351.56		4.18
Dry weight of seeds [3]	1	.65	4.58	242.00	
Number of pods	4	55.97	2.43	2.76	
Number of seeds [3]	1	5.34	4.22	242.00	
Dwarf milo; experiment 346:					
Dry weight of heads	2	29.51	9.68		8.02
Dry weight of tops without heads	2	2,232.86	17.19		8.02
Dry weight of roots	2	269.57	11.79		8.02
Dwarf milo; experiment 361:					
Weight of tops	4	7,626.38	67.25		4.89
Dry weight of roots	4	595.06	5.60		4.89

[1] Values of *F* equal to or exceeding these indicate that differences were statistically highly significant.
[2] Data restricted to cultures that received 0.02, 0.10, and 0.20 p. p. m. of zinc.
[3] Data restricted to cultures that received 0.10 and 0.20 p. p. m. of zinc. The differences have no statistical significance.

TABLE 3.—*The germination of peas produced by plants grown in nutrient cultures containing different quantities of zinc; experiment 267*

Beakers No.	Concentration of zinc at the start	Seeds planted	Seeds germinating
	P. p. m.	*Number*	*Number*
1–2–3	0	12	1
4–5–6	.005	15	12
7–8–9	.02	18	17
10–11–12	.10	19	16
13–14–15	.20	18	16

The fact that plants to which no zinc was supplied failed to produce viable seeds is, in itself, emphatic evidence that zinc is essential for the reproductive processes culminating in seed production. Although few seeds were produced when extremely minute amounts of zinc were furnished, their power of germination was good. This can be interpreted as evidence that zinc migrated from the vegetative organs of the plant and accumulated in the seeds.

This latter fact may explain why seeds from plants that received less than 1 part per 10 million of zinc did not differ greatly in viability. The presence of zinc in these peas, although in suboptimal amounts,

might nevertheless cause the formation of sufficient stored auxin to insure the development of the embryonic plants. Skoog (*10*) has demonstrated that the production of auxin is intimately connected with the presence of zinc.

BEANS

An experiment (No. 276) on the relation of zinc to the growth and seed production of Davis Dwarf Wax beans was begun on July 13. The nutrient solution and the concentrations of zinc were the same as those used in the preceding experiment with peas. There were thirty 2-liter beakers, each with three bean seedlings. Fifteen of the beakers were forcibly aerated, 15 nonaerated. On August 14 the beakers were emptied and refilled with nutrient solutions having the original composition. On September 5, owing to depletion of nitrates, there was added 5 cc. of $Ca(NO_3)_2$ molar solution to each beaker in the three lower concentrations, and 10 cc. of the same in the two higher concentrations. All plants began to form pods September 5, although the plants in the lowest concentrations were small. The plants in the 0.10-p. p. m. concentration of zinc were larger and produced more seeds than those in other concentrations.

The plants in experiment 276 were harvested September 26 and records were made of the dry weight, number of pods, number of seeds, etc. Several pods were formed in the minus-zinc aerated cultures, but none in the corresponding nonaerated cultures. No seeds were formed in any of the pods of plants in the minus-zinc cultures. In general the Dwarf Wax beans were better adapted for this type of culture experiment than the peas. The combined yields of aerated and nonaerated cultures are shown in figure 1, *B*, and in table 1, and their statistical reliability in table 2.

All plants in solutions having concentrations of 0.02 p. p. m. of zinc produced pods, but, with few exceptions, they were seedless. The plants in solutions having a concentration of 0.10 p. p. m. of zinc attained the best development, except with respect to leaf weight. In numbers of pods and seeds the plants in this concentration were decidedly superior, as shown by the fact that they produced about 35 percent of the total number of pods and 58 percent of the total number of seeds. The weights of leaves and roots were not, however, much greater than on the plants in 0.02 p. p. m. concentration.

There was a special value between 0.02 and 0.10 p. p. m. of zinc with respect to seed production. This indicates that a higher threshold level in concentration of zinc was needed for seed than for pod production. The root, seed, and pod production in 0.20 p. p. m. was not significantly different from that in 0.10 p. p. m.; the weight of leaves was somewhat greater, but the weight of roots was not significantly different.

Another experiment (No. 285) with Davis Dwarf Wax beans was set up August 11; the same concentrations of zinc as in the preceding experiments were used. There were 30 beakers in the experiment, all aerated, with three plants in each. On September 5, and again on October 10, potassium nitrate was added to the jars containing the larger amounts of zinc in which the plants had made greater growth and utilized more of the nutrient salts. On September 29 it was noted that superior growth was made by plants in the solutions containing the larger amounts of zinc, while in the solutions lacking zinc or containing only 0.005 p. p. m. growth was inferior,

as indicated by the small leaves and the dead margins of many of the older leaves. Many large bean pods, with many seeds, were found in the higher zinc concentrations. The plants were harvested on October 31.

In this experiment the plants showed a smaller range in the number of pods formed than in experiment 276. The weight of the pods, however, showed interesting relations to the concentration of zinc in the solutions. A statistical analysis (table 2) showed that the average number of pods in different cultures was not statistically significant, but their weight (a reflection of size) was highly significant. The number of seeds and their weight were strikingly related to the concentration of zinc in the nutrient solution, as shown by table 1 and figure 1, *C*. The existence of a special value for seed production, which was mentioned previously, was still more strikingly shown in the results of this experiment, where it appears that the special value lies between 0.02 and 0.10 p. p. m. of zinc. In this case the values of seed production in concentrations of 0.20 were not significantly different from those for 0.10 p. p. m. of zinc. This was determined not only by inspection of the graphs, but also by statistical analysis (table 2). The increased weight of tops and roots, corresponding to increasing concentrations of zinc, are quite uniform and parallel, showing no such threshold values in relation to the amount of zinc as in the case of seed formation. This corroborates evidence obtained from previous experiments showing that zinc has a very important role in the formation of fruits and seeds.

MILO

A similar experiment (No. 346) with dwarf milo was started April 24 and terminated August 23. After May 17 the culture solutions were artifically aerated. The original concentrations of zinc having been found inadequate for the milo plants, were supplemented by three subsequent additions which supplied a total of 0.02, 0.08, 0.40, and 0.80 p. p. m. Other nutrients were added on three occasions to replace the amounts that had disappeared.

When the plants in experiment 346 were harvested, August 23, it was noted that many in the solutions lacking zinc, as well as in the solutions of lower concentrations, had died sometime before harvest. All of those still alive were stunted and many showed signs of injury. Some of the plants showed the symptom known as "white bud," a common indication of zinc deficiency in maize and milo (*3*). It was only in initial concentrations of 0.1 and 0.2 p. p. m. of zinc that normal plants bearing heads were produced. The phenomenal increase in the weight of heads, as shown by figure 1, *D*, appeared between an initial concentration of 0.02 and 0.20. The dry weight of roots and tops increased as the amount of zinc increased. The probabilities that the differences were due to treatments are better than 99 to 1 (table 2), and indicate that zinc was therefore a very important factor in producing not only the vegetative plants, but also the reproductive heads.

A second experiment (No. 361) with dwarf milo in water cultures was started on June 27 and terminated October 16 (table 4). The zinc concentrations added were, none, 0.005, 0.02, 0.10, and 0.40 p. p. m., the last mentioned being given in four installments of 0.1 p. p. m. each. Additional KNO_3 was given August 2 and 16, and on

September 5 the beakers were emptied and refilled with fresh solutions. Plants that had received less than 0.1 p. p. m. grew very little and the majority were dead before the termination of the experiment. Since few of the heads contained seeds, their weights were not separately recorded. The differences in the weights of tops and roots (table 2) are highly significant, and show plainly the importance of zinc for the growth of this plant.

TABLE 4.—*Effect of the quantity of zinc supplied on the dry weight of dwarf milo plants grown in experiment 361*

[Average weight per jar of 3 plants]

Concentration of zinc supplied (p. p. m.)	Weight of—		Concentration of zinc supplied (p. p. m.)	Weight of—	
	Tops	Roots		Tops	Roots
	Grams	*Grams*		*Grams*	*Grams*
0	5.9	2.1	0.10	70.8	21.9
0.005	8.7	5.2	0.40	101.4	29.9
0.02	12.3	6.0			

SUMMARY

The necessity of zinc for the complete life cycle of plants is demonstrated by experiments in which highly purified nutrient salts were employed. Where no zinc was added, growth proceeded to a certain point but not far enough for seeds to develop.

There was a threshold value of zinc for peas and beans, below which the plants produced only small seedless pods. Above that value the pods were larger and contained seeds. The seeds produced were viable, although the parent plants received suboptimal supplies of zinc.

Milo showed definite symptoms of zinc deficiency in the lower concentrations supplied. There was a definite threshold value of zinc for the development of tops and heads.

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REVERSIONS IN MORPHOLOGY OF NITRITE-INDUCED "MUTANTS" OF ASPERGILLI GROWN ON AMINO ACIDS[1]

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INTRODUCTION

Evidence has been presented that variants or mutants of Aspergilli can be produced by the action of nitrous acid and other compounds destructive of amino groups.[2] Later it was reported that lysine or sodium thiosulfate acted to bring about reversion of these artificially produced forms toward the original morphology.[3] The suggestion was made that this type of mutation (injury) and reversion could be due to loss and recovery, respectively, of ϵ-amino groups in lysine units of the fungus proteins. In view of the considerable amount of evidence relative to interconversion of amino acids and possible transaminations, further tests were considered desirable.

EXPERIMENTAL TECHNIQUE

The nitrite-induced variants of the Aspergilli selected for additional study were the "yellow-woolly" strain (N1), obtained from *Aspergillus niger* Van Tiegh. (No. 4247), and a "woolly nonperithecial" strain, obtained from *A. amstelodami* (Mangin) Thom and Church (No. 126). These have already been described.[2] The former produces masses of mycelium with very few spores and has a yellow reverse. The latter forms an abundance of aerial hyphae covered with many green spores but with perithecia reduced to very few. They were grown in 200-cc. Erlenmeyer pyrex flasks on 50 cc. of base solution of the following composition: Water, 1,000.0 cc.; sucrose, 50.0 gm.; ammonium nitrate, 1.90 gm.; dipotassium phosphate, 0.35 gm.; and magnesium sulfate ($7H_2O$), 0.25 gm. Iron, zinc, copper, manganese, and molybdenum were added in concentrations of 0.20, 0.20, 0.05, 0.025, and 0.02 mg. per liter, respectively. The amino acids were added to this solution, as required, at a concentration of 10.0 gm. per liter, together with 20 gm. of calcium carbonate (1.0 gm. per flask). The *A. niger* (N1) cultures were kept at 35° C., the *A. amstelodami* (A1) at room temperature. Duration of growth was from 30 to 50 days. During this time transfers were made to Czapek agar slants at varying intervals to determine whether any variants had arisen.

In the experimental production of variants, transfers were made from any portion of the culture showing a contrast in morphology, particularly also from patches of sterile aerial hyphae. In attempts

[1] Received for publication September 5, 1941.

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to obtain reversions to type, transfers were made with spores of typical appearance whenever possible. Transfers were made first to Czapek agar slants and were continued with material from single heads until the cultures seemed to be pure.

The standards, or types, for estimating morphological variation were the original strains from which the stock variant cultures (N1, A1) had been isolated after being grown on sodium nitrite.

REVERSIONS WITH AMINO ACIDS

The results of studies on the ability of amino acids to cause reversions are summarized in table 1. These studies included trials (duplicate cultures) with the amino acids when used singly or in combination with lysine. A total of 38 variants was isolated after growth of the N1 strain of *Aspergillus niger* on the individual amino acids. Of these variants, 4 were typical *A. niger* strains, and 23 were more nearly typical in appearance than the N1 strain. In admixture with lysine, 44 variants were produced of which 6 resembled the original strain very closely in appearance and 29 resembled it more closely than did the N1 strain. One of the strains isolated from an N1 culture containing lysine together with histidine was found on comparison to be practically identical with the mutant obtained by Schiemann [4] with potassium bichromate and named *A. cinnamomeus* because its spores were brown instead of black.

The corresponding totals for *Aspergillus amstelodami* (A1) without lysine were 18 variants, of which none exactly resembled the standard strain, but 16 had a closer resemblance than was shown by the A1 strain. In combination with lysine, 23 variants were isolated, of which 1 was a typical *A. amstelodami* and 19 were less atypical than the A1 strain. The single typical *A. amstelodami* strain was obtained following growth on a mixture of lysine and threonine. Its isolation partook of the nature of a fortunate chance, since the growth was small and quickly submerged. Its identification was possible only because of the system of daily examinations employed.

The differences between the use of amino acids with and without lysine are definitely in favor of the admixtures in aiding reversions. The figures are not impressive, however, as indicative of a marked variation in effectiveness. Nevertheless these values do not reflect the extent to which reversions seemed to have taken place in the two series in the individual isolated strains. On this basis there could be little question of the superiority of admixture with lysine. Complete reversion to standard forms occurred in the absence of an amino acid supply in one instance when addition of calcium carbonate was omitted. In the presence of calcium carbonate the N1 and A1 strains produced no variants without a supply of amino acid.

The reverted strains that were isolated from cultures of the "yellow-woolly" strain of *Aspergillus niger* varied considerably in appearance though many of the cultures seemed to be quite similar. The reverse of the mycelial felts varied from white to black, to pink, and to chocolate. The quantity of spores and aerial hyphae also varied greatly. The standard or typical strain was black with spores, aerial hyphae were practically absent, and the reverse of the mycelial felts was usually light tan.

[4] SCHIEMANN, ELISABETH. MUTATIONEN BEI ASPERGILLUS NIGER VAN TIEGHEM. Ztschr. f. Induktive Abstam. Vererbungslehre 8: 1-35, illus. 1912.

TABLE 1.—*Variants obtained from cultures of nitrite-induced mutants of Aspergillus niger and A. amstelodami after growth on amino acids*

Amino acid (10.0 gm. per liter)	Variants with *Aspergillus niger* "yellow-woolly" strain (N1)						Variants with *Aspergillus amstelodami* "woolly nonperithecial" strain (A1)					
	Minus *d*-lysine			Plus *d*-lysine			Minus *d*-lysine			Plus *d*-lysine		
	Total	Reversion complete	Reversion incomplete	Total	Reversion complete	Reversion incomplete	Total	Reversion complete	Reversion incomplete	Total	Reversion complete	Reversion incomplete
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
dl-Alanine	1	0	1	1	0	1	0	0	0	1	0	1
d-Arginine hydrochloride	3	0	2	6	1	4	1	0	1	2	0	2
l-Aspartic acid	2	0	2	2	1	0	0	0	0	1	0	1
l-Cystine	2	1	1				1	0	1			
d-Glutamic acid	2	0	1	1	0	1	1	0	1	1	0	1
Glycine	2	0	1	2	0	1	1	0	1	1	0	1
l-Histidine dihydrochloride	3	0	1	2	[1] 2	0	2	0	2	2	0	2
l-Hydroxyproline	1	0	0	1	0	0	1	0	0	1	0	0
dl-Isoleucine	2	0	2	5	0	4	1	0	1	1	0	1
l-Leucine	0	0	0	2	0	2	2	0	2	1	0	1
d-Lysine dihydrochloride				2	0	2				1	0	1
dl-Methionine	0	0	0	2	0	2	0	0	0	1	0	1
dl-Norleucine	2	0	1	2	0	0	0	0	0	1	0	1
dl-Phenylalanine	2	0	1	1	0	1	0	0	0	0	0	0
dl-β-Phenyl-β-alanine	3	1	2	2	0	2	2	0	2	1	0	1
l-Proline	0	0	0	0	0	0	0	0	0	2	0	0
dl-Threonine	4	1	3	3	0	3	1	0	1	2	1	1
l-Tryptophane	3	0	3	4	0	3	1	0	1	1	0	1
l-Tyrosine	1	0	0	2	1	1	1	0	1	0	0	0
dl-Valine	2	1	1	2	1	1	2	0	2	1	0	1
Control [2]	0	0	0				0	0	0			
β-Alanine	3	0	1	1	0	0	1	0	0	1	0	1
Cysteine hydrochloride				1	0	1				1	0	1
Total	38	4	23	44	6	29	18	0	16	23	1	19

[1] 1 of these strains was identified as *Aspergillus cinnamomeus*.
[2] No amino acid.

Changes of a similar nature took place with *Aspergillus amstelodami*. The most marked of these changes were associated with enhancement of perithecial formation. Interesting atypical forms also occurred that produced a broadly spreading, more or less woolly colony, upon which short conidiophores were produced in abundance along trailing hyphae and ropes of hyphae. These conidiophores were short, the heads small; the whole colony became dark green; perithecial development was inhibited or suppressed; and none of the orange color so characteristic of the species was evident.

The great frequency with which apparently identically reverted forms were isolated gave the impression that, while experimental manipulation of environmental conditions influenced these changes, the more important factor was the internal mechanism of the organism. Internal instability might be increased by amino acids and perhaps guided somewhat in the direction of the original morphology, but the changes that occurred were in all directions within the limits of the species. It is quite possible, on the other hand, that continued investigation along these lines may lead to a better understanding of the mechanisms involved and the development of reagents of much greater specificity.

Results obtained in the parallel series of table 1 are complicated by the fact that the strains selected for use did not remain entirely un-

changed. The "yellow-woolly" strain of *Aspergillus niger*, after 23 transfers during 3 years, no longer was yellow on its reverse side and aerial hyphae had become profuse, whereas the *A. amstelodami* strain became completely free from perithecia during this interval. The change with *A. niger* began on the twenty-first transfer. The further adjustments in morphology of these atypical strains, or "injury mutants," were in a direction toward greater abnormality and seemed to be accompanied by a greater resistance to the action of the amino acids.

Each strain was tested once with the following miscellaneous organic compounds. Amino acids were not added. Only with nicotinic acid did reversion to a typical strain occur in *Aspergillus niger*. Changes toward complete reversion (spores) were also exhibited by strains isolated from cultures with pyrazine-2,3-dicarboxylic acid; 2-methyl-1,4-naphthoquinone; calcium *d*-pantothenate; biotin concentrate; β-carotene; ascorbic acid; thiamin chloride; and *i*-inositol. Loss of yellow color had taken place in strains isolated from cultures with pyrazine monocarboxylic acid, calcium pantothenate, biotin concentrate, pyruvic acid, ascorbic acid, and *i*-inositol. No forms of differing characteristics could be isolated from cultures containing phthiocol, α-tocopherol, sodium hyposulfite, lecithin (egg), nicotinic acid amide, sodium-iron chlorophyllin, riboflavin, pimelic acid, and vitamin B_6.

In the *Aspergillus amstelodami* series partially reverted strains were obtained with biotin concentrate, α-tocopherol, pimelic acid, vitamin B_6, and thiamin chloride. All were similar in appearance and characterized by an abundance of perithecia, spores, and aerial hyphae. The original or standard strain was practically free from aerial hyphae.

It seemed possible that through the use of admixtures additional evidence might be obtained as to whether internal instability or determinative influence of amino acids was the predominant factor in producing these results. That is to say, a mixture of amino acids and accessories, in the presence of each of whose constituents a reversion to standard had taken place, should give results much superior to those obtained with the individual compounds. This would not follow invariably, since mutual inhibition might occur but should not take place in every instance. Results with admixtures followed the usual pattern, ranging from recovery of the mutant only to recovery of strains showing complete reversion. The best results were given by a mixture of *d*-lysine, *dl*-valine, and nicotinic acid with the *Aspergillus niger* mutant. Six variants were obtained from a single culture, including (1) reversion complete, smooth white reverse, (2) reversion complete, rough white reverse, (3) reversion complete, black reverse, (4) reversion complete, red to chocolate reverse, (5) white woolly with many spores, and (6) woolly, almost normal sporulation, smooth pink reverse. The substitution of tyrosine for valine led to the isolation of an almost fully reverted strain with red to chocolate reverse. Calcium carbonate was not added to these cultures. Results with mixtures were negative with *A. amstelodami* (A1), and gave poorer results than usual, since not a single strain showing any degree of reversion appeared.

UTILIZATION OF AMINO ACID NITROGEN

It seemed desirable in conclusion to compare the growth responses of a variant to amino acids with those of the original or standard strain. To do so might throw some light on the cause of the action of amino acids in favoring reversions, and on a possible relation between capacity for amino acid assimilation and normalcy. The "yellow-woolly" strain of *Aspergillus niger* was selected for this purpose because the amino acid nutrition of the standard strain had already been studied.[5] Data on a third strain, "slightly atypical," were also obtained (table 2). This had been isolated from stock cultures of the standard strain after continued propagation for several years on a medium containing 1 percent each of peptone, sucrose, and agar, together with traces of Difco peptone and malt extract. It was characterized by lower yields, greater acidification of the substrate, and higher starch production. In appearance it resembled the standard strain very closely when grown on Czapek agar.

TABLE 2.—*Utilization of nitrogen sources by a standard strain of Aspergillus niger and by 2 of its "mutants" during 4, 5, and 11 days of growth at 35° C.*

Nitrogen source	Standard strain [1]		Slightly atypical strain		"Yellow-woolly mutant" (N1)			
	Yield after 4 days	Sporulation [2]	Yield after 4 days	Sporulation [2]	Yield after 5 days	Sporulation [2]	Yield after 11 days	Sporulation [2]
	Mg.		*Mg.*		*Mg.*		*Mg.*	
dl-Alanine	1,115.8	10	596.7	4	160.0	0	552.0	0
d-Arginine hydrochloride	1,252.4	10	968.0	4	150.9	0	520.4	0
l-Aspartic acid	1,325.1	10	1,130.0	6	209.9	0	611.4	0
l-Cystine	71.0	1	70.5	1	108.5	0	255.3	0
d-Glutamic acid	1,292.8	10	1,196.5	10, bb	187.6	0	524.5	0
Glycine	1,234.8	10	933.8	4	115.0	0	503.6	0
l-Histidine dihydrochloride	120.9	4	213.9	4, t	73.7	0	591.7	0
l-Hydroxyproline	1,147.4	10	747.3	2	5.2	0	44.9	0
l-Iodogorgoic acid	0	0						
dl-Isoleucine	246.2	4	84.8	0	42.8	0	270.5	0
l-Leucine	373.6	6	160.1	0	11.0	0	326.4	0
d-Lysine dihydrochloride	16.2	0	14.7	1	8.1	0	196.6	0
dl-Methionine	437.0	7	273.2	0	0	0	414.8	0
dl-Norleucine	113.8	1	91.0	0		0	223.6	0
dl-Phenylalanine	370.8	4	206.0	1	2.7	0	280.8	0
l-Proline	1,110.7	10	1,019.0	8	115.2	0	531.9	0
dl-Serine	608.6	7	723.2	0	83.9	0	384.7	0
dl-Threonine	647.4	6	345.5	0	95.1	0	386.3	0
l-Tryptophane	636.3	6	555.9	2			351.4	0
l-Tyrosine	196.1	10	227.9	6	110.0	0	310.9	0
dl-Valine	291.8	1	186.9	0	80.4	0	315.9	0
Cysteine hydrochloride	12.2	0	10.2	0			21.2	0
d-Alanine	18.7	4	29.5	3, br				
Ammonium nitrate	1,213.2	10	709.8	2	139.7	0	677.5	2

[1] Data from Steinberg (see footnote 5).

[2] Sporulation is rated from 0 (sterile) to 10 (covered with spores). Where spores were other than a normal black color, they are indicated by bb (black and brown), br (brown), and t (tan).

The responses obtained respecting utilization of amino acids would indicate that incapacity for normal development of the variant strains is accompanied by a similar incapacity to employ these acids in a normal manner. Inhibition in the utilization of amino acid nitrogen is relatively greater with aspartic acid, glutamic acid, and proline. These are the fully plastic acids, so-called because best suited to supply both nitrogen and carbon to the standard organism. The

[5] STEINBERG, ROBERT A. EFFECT OF TRACE ELEMENTS ON GROWTH OF ASPERGILLUS NIGER WITH AMINO ACIDS. Jour. Agr. Res. 64: 455-75.

relatively higher yields with the practically unassimilable or aplastic acids (cystine, histidine, lysine, norleucine, and tyrosine) are probably due to some extent to an incapacity to employ the plastic acids. The "yellow-woolly" strain had lost the capacity to utilize hydroxyproline, whereas histidine sufficed to give maximum yields. It had also become a high producer of starch. The immediate causes of these altered responses will probably be found in a disturbance of the basic enzymatic complement of the original organism from which this "injury mutant" was obtained with nitrite. The presence, for example, of a greater content of histidase in the variant as compared to the original strain could lead to these results. The glutamic acid and ammonia formed from histidine would give improved growth. An interesting phase of this question is concerned with the possibility of so causing formation of new enzymes. Such changes perhaps occur but are still to be demonstrated.

On the whole, however, the distinguishing features of the amino acid responses by the standard strain are also recognizable in the aberrant strains. Those acids best assimilated by the standard strain were among those best assimilated by the variant strains. Those most poorly utilized by the standard strain were among those least readily made use of by the variant strains. In general, both variant strains exhibited a decreased capacity to employ the fully plastic amino acids and an increased capacity to use the aplastic acids. These modifications may be the underlying cause for their decreased rate of growth. The degree to which modifications in amino acid responses were obtained corresponded broadly with the extent to which morphological alterations had occurred in the mutants. The deterioration in capacity of the slightly atypical strain to employ inorganic nitrogen as compared to organic nitrogen is indicative perhaps of the possibility of producing a strain of *Aspergillus niger* incapable of assimilating inorganic nitrogen. Such a change has been accomplished with bacteria.

DISCUSSION

Experiments having for their purpose the study of the action of a supply of normal cell constituents can be considered to a great extent as concentration studies. At no time can these compounds be viewed as completely absent from the organism. It is not strange, therefore, that experimental reversions not only are induced through addition of amino acids to the substrate but also occasionally arise spontaneously. Abnormal metabolism of cells, leading to localized accumulations of the individual amino acids, may be a factor in the occurrence of such spontaneous reversions. Tryptophane has been found capable of causing the formation of galls in green plants.[6] Whether amino acids are the primary cause of these variations or are effective only indirectly remains to be determined.

The evidence gathered in trials with the amino acids would appear to indicate that reversions may arise in the presence of other amino acids than lysine. Though the data on which a special role for lysine was assumed is thereby weakened, this amino acid is not entirely eliminated as a possible primary factor. Its presence is conducive to best results, whether its action be direct, as formerly postulated, or

[6] KRAUS, E. J. HISTOLOGICAL REACTION OF BEAN PLANTS TO L-TRYPTOPHANE. Bot. Gaz. 102: 602–622, illus. 1941.

indirect through interconversion or transamination. No data are available at the present time on the ability of amino acids to serve in vitro for replacement of amino groups in deaminized proteins.

Reversions to the original morphology took place with the *Aspergillus niger* N1 strain when grown on lysine, cystine, β-phenyl-β-alanine, threonine, and valine; or in admixture with lysine, on arginine, aspartic acid, histidine, tyrosine, or valine. A mixture of nicotinic acid, lysine, and valine gave still better results. The *A. amstelodami* A1 strain gave reversion to the original morphology only with a mixture of lysine and threonine. It is doubtful, therefore, whether nitrogen nutrition as ordinarily conceived will be found to be the immediate cause of this phenomenon. Excepting arginine, histidine, and aspartic acid, the nine acids most effective in bringing about reversions are least efficient as sources of nitrogen for the N1 strain of *A. niger*.

Intensive studies with a single variant on a much larger scale than was feasible in these investigations should lead to a clearer answer concerning the process of inheritable variations. Larger scale experiments would afford material for statistical evaluation and so help to distinguish between spontaneous and induced changes. Accumulation of additional data with at least every possible combination of amino acids in pairs is advisable. Nor should other cell metabolites be neglected inasmuch as the available facts would indicate that the primary cause of these alterations is injury to the mechanism of cell metabolism. However, no further studies in this field are contemplated by the writers, since conditions make necessary their discontinuance.

Information on the loci of injury is so slight that only speculation is possible on this phase at this time. The data indicate that variations in enzymatic content have taken place, and perhaps some formed de novo. A study of the shifts observed in the normal pattern of amino acid utilization accompanying inheritable structural variations due to injury should prove a valuable tool in morphological investigations. They point to a direct interrelation between the enzymatic complement of the organism and morphological normalcy. This correlation is not unexpected, since inheritance must have its fundamental basis in nutrition, and this study and those of earlier workers would substantiate this interpretation. Studies of this type, therefore, provide a means for obtaining direct information concerning the relation of metabolic capacities to structure and development and of their bearing on inheritable variations. Cell maturation in animals is also interfered with by carcinogenic compounds.

SUMMARY

Reversions to the morphology of the original strain took place with a nitrite-induced injury-mutant of *Aspergillus niger* Van Tiegh. when grown on lysine, cystine, β-phenyl-β-alanine, threonine, and valine; or in admixture with lysine, on arginine, aspartic acid, histidine, tyrosine, or valine. A mixture of nicotinic acid, lysine, and valine gave the best results. A nitrite-induced injury-variant of *A. amstelodami* (Mangin) Thom and Church gave complete reversion to the morphology of the original strain only with a mixture of lysine

and threonine. Assimilability of amino acids did not appear to be a major factor in these responses.

Comparison of capacities for amino acid utilization between the standard strain and each of two variant strains of *Aspergillus niger* disclosed alterations proportional to the extent of morphological change. These changed assimilation capacities in the variants did not destroy the general nature of the responses characteristic of the original strain, though individual amino acids were affected. The variant having greater alterations in morphology showed much less capacity for assimilation of the fully plastic acids (aspartic acid, glutamic acid, and proline) and increased capacity for utilization of the aplastic acids (cystine, histidine, lysine, norleucine, and tyrosine). Utilization of hydroxyproline had become particularly poor in the almost sterile or more atypical variant. Enzyme suppression with nitrite disturbed cell maturation as in abnormal cells produced in animals by carcinogenic compounds.

MICROBIOLOGICAL AND NUTRITIONAL FACTORS IN THE TAKE-ALL DISEASE OF WHEAT[1]

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INTRODUCTION

Numerous soil amendments (*3*, *5*, *8*)[3] as well as tillage practices (*13*) have been reported of value in controlling the take-all disease of wheat (*Triticum aestivum* L.), caused by *Ophiobolus graminis* Sacc. In many instances procedures effective in one locality are ineffective in another, or even in the same region or field in different years. The spontaneous disappearance of diseased areas in fields has been noted (*4*). The extent to which manurial and tillage practices are effective and the precise mechanism of their influence on the incidence of the disease remain controversial. Organic amendments have been generally successful in providing control even under greenhouse conditions, where some treatments effective in the field fail. It has been suggested (*3*, *5*, *11*, *14*) that organic amendments reduce the severity of take-all disease because they stimulate antagonistic or competitive micro-organisms in soil.

Previous work at this laboratory (*1*, *2*) has shown that organic amendments applied to soil have no apparent effect on root-surface microfloras, even though markedly stimulating the soil microflora. It was suggested that, once the root parasite reaches the susceptible plant, factors of nutrition, host resistance, or parasite virulence are probably more important than microbial antagonisms. But nutritional aspects of organic manures have been considered of little importance. It has been stated that the effects of animal manures are due to their action on the parasite and not to their benefits to the host plant (*10*). Soils of the eastern-central region of Kansas in which take-all disease occurs are frequently low in available phosphorus (*16*), and phosphatic fertilizations have at times been sufficient to control take-all in the field (*8*, *13*).

Garrett (*7*) has suggested that the presence of a suitable nitrogen supply plays an important role in permitting the survival of *Ophiobolus graminis* in soil, and in recent work he considered competition among soil micro-organisms for nitrogen more significant than antagonism. He stated (*6*) as characteristic of organic manures that control take-all that they contain insufficient or just sufficient nitrogen for their de-

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[2] Now included in the Division of Soil and Fertilizer Investigations.

[3] Italic numbers in parentheses refer to Literature Cited, p. 665.

composition. This suggests that the amount of available nitrogen as well as of available phosphorus may be of importance in the incidence or disappearance of *Ophiobolus*. The present investigations were designed to evaluate the importance of microbial activity and of available-phosphorus and nitrate-nitrogen levels of soil as determining factors in the incidence or severity of take-all disease.

METHODS AND MATERIALS

Both field areas and potted experimental soils were employed for study; the greenhouse experiments were established in order to provide more pronounced variations in the factors under study than might be readily encountered under field conditions. Field areas that had histories of take-all disease for several years, and adjacent areas in which take-all damage had not been observed, were selected for study. Soil samples for analyses were collected at intervals throughout several crop seasons and consisted of well-mixed aliquots, as nearly free of roots as possible, taken from the upper 4 to 6 inches of soil at several sampling stations. From certain naturally infested field areas, soil was collected also in larger quantities for greenhouse experiments; for this purpose no attempt was made to avoid the inclusion of diseased root fragments. Each soil lot collected for potting was sieved and thoroughly mixed to insure uniformity in the subsequent aliquot portions required.

The methods employed for determination of numbers and types of micro-organisms have been reported previously (*1*, *2*). The method of Truog and Meyer (*17*) was used in the determination of available phosphorus, and the phenoldisulfonic acid method, as modified by Harper (*9*), in the determination of nitrate nitrogen. The pH values, as determined with a glass electrode, of all soils employed fell between 5 and 7. All colorimetric readings were made with a Duboscq colorimeter.

FIELD OBSERVATIONS

For take-all infested and noninfested field areas of the silt loam and clay loam prairie soils studied, differences either in numbers or kinds of micro-organisms were not apparent. The plate-count estimates of either total micropopulations, or of component subgroups thereof, as actinomycetes, filamentous fungi, total bacteria, or dye-tolerant, fluorescent, and spore-forming types of bacteria, showed close agreement on any given date of sampling. To exemplify, bacterial numbers averaged 28.7 million per gram of air-dry soil in eight infested areas and 25.1 million per gram in eight noninfested areas sampled June 14, 1938. Complete seasonal studies showed that at no time was it possible to identify infested areas on the basis of microbial numbers found therein. The isolation and examination of single cultures showed the same bacterial types and species predominant in infested as in noninfested soil; striking differences, such as are evident between soil and root-surface microfloras, could not be observed. Those microbial species commonly recorded as antagonistic in plate culture or pot experiments, as *Bacillus vulgatus*, *Trichoderma* spp., and *Actinomyces* spp., could be isolated with equal facility from either soil condition.

Available-phosphorus levels of take-all infested and noninfested field areas, as revealed by soil samples collected in May and June

1940, are shown in table 1. Although no correlation between the level of available phosphorus and the presence or absence of disease was apparent, it was noted that the available-phosphorus level was 18.3 parts per million of phosphorus in one field for which there was a history of reduction in the severity of take-all disease following phosphatic fertilization.

TABLE 1.—*Available-phosphorus levels in soil of take-all areas and in immediately adjacent field portions or fields on which healthy wheat was grown*

Sample pair No.	Available phosphorus in—		Sample pair No.	Available phosphorus in—	
	Infested area	Noninfested area		Infested area	Noninfested area
	P. p. m.	*P. p. m.*		*P. p. m.*	*P. p. m.*
1	67.7	69.8	17	31.4	37.2
2	14.3	13.3	18	14.8	17.6
3	19.2	21.2	19	23.7	21.0
4	75.6	51.5	20	44.3	61.4
5	23.2	26.9	21	38.5	58.1
6	14.1	12.7	22	25.6	36.1
7	18.3	18.3	23	103.6	30.8
8	14.0	18.8	24	21.2	23.2
9	25.8	9.0	25	16.4	21.0
10	23.6	20.5	26	20.0	19.0
11	22.4	28.8	27	38.9	28.3
12	41.3	29.2	28	24.2	14.0
13	130.2		29	44.1	47.7
14	41.2	20.4	30	26.0	33.8
15	53.8	49.7	31	12.3	12.5
16	38.0	30.5			

EXPERIMENTAL WORK

EXPERIMENT 1

Naturally infested silt loam was divided into 18 aliquot portions, and each portion was subsequently treated as outlined in table 2. A total of 54 pots, 3 from each soil portion, was established by potting 1,500-gm. amounts in unglazed 6-inch clay pots. Soil was brought to optimum moisture content October 2, 1939, and water was added daily in amounts sufficient to keep the soils near such content until the termination of the experiment. Kanred wheat was planted October 6, and 3 plants were retained in all except 3 pots (soil aliquot 18), which were fallowed throughout the experiment.

Soil samples were removed from each of the 54 pots on October 6 and 27, November 28, January 12, and March 13, of the 1939–40 season. Replicate samples taken October 27 and November 28 were handled separately for microbiological analyses; those of the other dates were composited thoroughly and then treated as single samples. Data concerning the microbial populations on October 27 and November 28 were sufficient to permit analysis for differences in microbial numbers due to soil treatment. A summary of the microbiological data, together with initial soil treatments, is given in table 2.

For chemical analyses, the replicate samples collected on each of the five dates were composited. Laboratory analyses of these composites revealed that maximum differences from the check soil portion were obtained, as might be expected, with maximum applications of the amendments employed. The completion of the experiment revealed (1) that those soil-nutrient conditions usually considered

TABLE 2.—*Influence of soil treatments employed in experiment 1 upon the soil microbial populations of naturally infested silt loam as revealed by samplings, after growing Kanred wheat, 1939*

Soil aliquot No.	Rate of treatment with—			Micropopulations determined per gram of air-dry soil									
	Superphosphate	Potassium nitrate	Ground wheat straw	Total bacteria		Dye-tolerant bacteria		Spores of aerobic bacteria		Actinomycetes		Filamentous fungi	
				October 27	November 28	October 27	November 28	October 27	November 28	October 27	November 28	October 27	November 28
	P. p. m.	*P. p. m.*	*P. p. m.*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*
1				64.7	28.8	28.0	4.6	8.1	2.5	7.3	6.7	0.26	0.16
2	25			77.3	33.9	35.7	6.4	9.7	3.7	19.3	7.1	.38	.13
3	50			71.9	38.8	24.3	9.5	*13.5	**4.9	9.5	4.9	.17	.14
4	100			88.8	38.8	25.8	7.7	8.0	2.8	17.4	8.0	.24	.15
5			1,000	90.2	39.9	34.1	*11.0	7.5	2.9	*21.6	9.4	.35	.24
6			2,500	**165.6	**55.6	**85.8	*10.3	8.7	3.6	**24.8	*10.9	**.67	**.47
7			5,000	**189.2	**76.4	**120.5	**27.1	*13.2	**5.8	15.5	*12.3	**.93	**.68
8		40		62.6	37.5	18.8	6.1	6.5	3.0	14.5	7.7	.25	.19
9		80		72.9	39.0	24.0	6.2	6.2	3.7	*20.3	9.4	.24	.19
10		160		65.4	29.6	30.8	6.7	8.4	*4.0	12.1	6.5	.27	.15
11	25	80		68.1	30.4	10.8	6.2	7.1	3.6	15.8	8.3	.30	.27
12	50	40		107.9	*44.4	25.1	**12.8	8.9	3.5	8.0	8.7	.33	.19
13	50		1,000	94.6	42.7	44.5	8.3	11.3	3.3	*20.4	*11.6	.49	*.34
14	25		2,500	91.1	*57.4	**63.8	**15.2	8.1	3.7	*22.3	*12.1	*.56	**.44
15	25	40	1,000	93.4	43.8	43.2	*10.6	9.7	3.3	*23.3	8.5	.35	.23
16		80	1,000	90.4	**50.1	38.5	*10.2	8.3	3.8	**27.5	*11.3	.26	.27
17		40	2,500	**165.0	**55.7	**66.9	**12.6	7.6	3.0	**27.9	*11.5	*.56	**.46
18				91.6	30.6	33.1	3.9	7.4	3.2	*22.9	8.9	.22	.22
Minimum corresponding value:													
Significant				113.6	44.2	47.7	10.0	12.9	4.0	19.7	10.9	.56	.31
Highly significant				130.3	49.5	54.5	11.8	14.6	4.5	23.9	12.4	.66	.36

*Value exceeds the minimum corresponding value, which is significant; **value exceeds the minimum corresponding value, which is highly significant.

satisfactory for plant growth were not maintained until the wheat matured, even with the heaviest rates of fertilizer applications employed, and (2) that all pots contained badly diseased wheat at maturity. Accordingly, only the chemical analyses of the check soil and the portions receiving the heaviest fertilizer applications need be considered.

The highest available-phosphorus values (27.5, 41.9, 29.6, and 25.5 p. p. m. of phosphorus) for the check and for the heaviest superphosphate, potassium nitrate, and wheat-straw fertilizations, respectively, were observed in the initial sampling of the experiment, and the lowest values (20.5, 29.7, 20.0, and 18.9 p. p. m. of phosphorus) were obtained in the final sampling on March 13. Although the soil that received the heaviest superphosphate fertilization maintained the highest available-phosphorus level throughout, even this level fell to a rather low value during the latter part of the season. The highest nitrate-nitrogen values observed during the fall for the four soil portions named were 13.6, 14.9, 40.2, and 8.0 p. p. m. of nitrogen, respectively, and the lowest, obtained March 13, were 2.0, 3.8, 5.0, and 0.6 p. p. m. Though distinct differences in the nitrate content of the soils existed early in the fall, during the latter half of the experiment all plants showed the yellowing characteristic of nitrogen starvation. The generally low values revealed by the March 13 samples also indicated that plant growth was limited because of low levels of available nitrogen.

During the first week of April 1940, all wheat plants were removed from pots, and the root systems were washed clean. The roots, crowns, and culms of all plants were severely diseased. But since for a considerable period the phosphorus and nitrate-nitrogen content could not be considered as satisfactory, no assumption could be made that plant-nutrient factors were relatively unimportant, even though initial fertilizer applications had been made at rates superficially comparable to those employed in the field. A second experiment was clearly indicated in which different levels of available phosphorus and nitrogen would not only be provided initially but would also be maintained throughout the growing season.

EXPERIMENT 2

Naturally infested silt loam was divided into 12 aliquot portions for treatment. Superphosphate (45 percent P_2O_5) was added at rates of 50, 100, 200, 600, and 1,000 p. p. m. to soil portions receiving no nitrogen treatment, and at these same rates to soil portions receiving, by the end of the experiment, a total of 976 p. p. m. of nitrogen. For determining this latter rate, the approximate amount of nitrogen required for plant growth was calculated, and inorganic nitrogen (two-thirds as potassium nitrate and one-third as ammonium nitrate) was added to provide nitrogen slightly in excess of the calculated figure. The initial application consisted of 40 p. p. m. of nitrogen, and subsequent additions averaging 16 p. p. m. were made at intervals during the growing season, so that by March 11 a total of 350 mg. of nitrogen per wheat plant had been added (table 3).

Initial soil treatments were made September 19, 1940, and two unglazed pots were filled from each soil portion. All containers were then brought to optimum moisture content. Kanred wheat was

seeded September 23, and three plants were retained in each pot. All soils were sampled September 23 and November 13, and the individual samples were analyzed microbiologically. No statistically significant differences were found in saprophytic micropopulations following the inorganic treatments employed (table 4). Further microbiological analyses were considered unnecessary. Complete samples for chemical analyses were taken September 23 and November 13, 1940, and March 20, 1941, and occasional random samples between November 13 and March 20, to check on nitrogen levels only. Nitrate-nitrogen and available-phosphorus levels for the first and last dates of sampling are given in table 3.

TABLE 3.—*Nitrogen and phosphorus applied in fertilizer, plant growth obtained, and severity of take-all damage to Kanred wheat grown in naturally infested silt loam in experiment 2*

Soil aliquot No.	Superphosphate (45 percent P_2O_5) applied	Inorganic nitrogen applied per plant	Oven-dried plant material per pot		Observed severity of take-all damage
			Top growth	Root system	
	P. p. m.	*Milligrams*	*Grams*	*Grams*	
19	50		1.93	0.65	Severe.
20	100		2.08	.53	Do.
21	200		1.95	.53	Do.
22	600		2.30	.50	Do.
23	1,000		2.30	.75	Do.
24			2.10	.90	Do.
25		330	11.10	.90	Do.
26	50	350	14.90	2.63	Moderate.
27	100	350	18.00	2.98	Do.
28	200	350	21.35	4.85	Slight.
29	600	350	34.15	6.33	Very slight.
30	1,000	350	35.30	7.05	None.

On March 20, 1941, all plants were removed, and the root systems were washed free of soil, care being taken to keep as many roots as possible attached to the plants. Clean roots and plants were examined for disease lesions, and oven-dried plant material was weighed. Summaries of nitrogen and phosphorus added, weights of plant materials (tops and roots separately), and comparative severity of take-all damage are given in table 3.

Weights of root material recovered by the procedure given and the severity of plant damage by disease showed a close relationship. Roots of all plants were severely diseased (fig. 1), whether grown in untreated soil, in soil that had received either low or high rates of superphosphate without nitrogen, or in soil that had received nitrogen treatment without superphosphate. The total weight of attached root material recovered from such soil treatments (soil aliquots 19 to 25, inclusive, table 3) was less than 1 gm. per pot, as compared with 7 gm. where control was obtained (soil aliquot 30). An adequate supply of either available phosphorus or nitrate nitrogen alone appeared incapable of effecting an appreciable reduction in the severity of the disease.

The weights of root systems recovered from naturally infested soil supplied with sufficient nitrogen showed direct correlation with the supply of available phosphorus. The beneficial effect of increasingly higher levels of available phosphorus in reducing disease became apparent when nitrogen supply was not a limiting factor (table 3;

TABLE 4.—*Influence of different levels of available phosphorus and nitrogen (see table 3) employed in experiment 2 upon the soil microbial populations of naturally infested silt loam and the soil content of available phosphorus and nitrate nitrogen after growing Kanred wheat, 1940–41*

Soil aliquot No.	Micropopulations determined [1] per gram of air-dry soil							Available phosphorus		Nitrate nitrogen	
	Total bacteria		Dye-tolerant bacteria		Spores of aerobic bacteria		Actinomycetes		Filamentous fungi		
	Sept. 23, 1940	Nov. 13, 1940	Sept. 23, 1940	Nov. 13, 1940	Sept. 23, 1940	Nov. 13, 1940	Sept. 23, 1940	Nov. 13, 1940	Nov. 13, 1940	Sept. 23, 1940	March 20, 1941	Sept. 23, 1940	March 20, 1941
	Millions	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*Millions*	*P. p. m.*	*P. p. m.*	*P. p. m.*	*P. p. m.*
19	53.0	88.2	26.9	15.9	3.8	4.0	8.4	7.6	0.27	33.1	22.5	14.4	0
20	52.8	73.4	27.0	12.5	3.3	4.1	5.0	8.0	.20	43.9	26.7	19.3	0
21	52.5	72.3	35.3	13.7	3.6	4.2	6.2	12.1	.18	58.6	41.7	14.4	0
22	47.5	76.0	18.1	12.1	3.4	4.3	6.2	8.2	.18	125.2	79.5	17.8	0
23	34.3	101.0	16.9	15.4	3.3	3.7	8.1	15.0	.24	196.0	85.3	20.3	0
24	50.0	65.3	13.5	10.1	2.7	4.8	8.3	7.6	.23	24.8	17.2	9.3	0
25	38.4	71.4	11.2	8.1	2.2	2.4	7.6	9.6	.32	23.5	17.0	5.0	16.3
26	54.0	83.1	13.3	9.4	3.2	3.5	9.0	8.4	.23	33.6	16.9	33.8	23.3
27	55.0	81.3	12.5	12.4	3.3	3.8	5.9	8.0	.26	42.5	18.4	35.6	12.3
28	43.7	107.0	14.8	13.3	2.4	3.1	7.4	8.5	.25	57.0	22.9	19.8	2.8
29	50.6	95.7	23.1	19.1	2.8	5.4	7.0	8.5	.34	132.2	53.5	34.0	3.2
30	56.3	104.4	8.9	16.5	2.4	4.2	9.5	8.9	.27	197.4	74.4	37.2	.4

[1] Statistical analyses of micropopulations recorded revealed no significant differences.

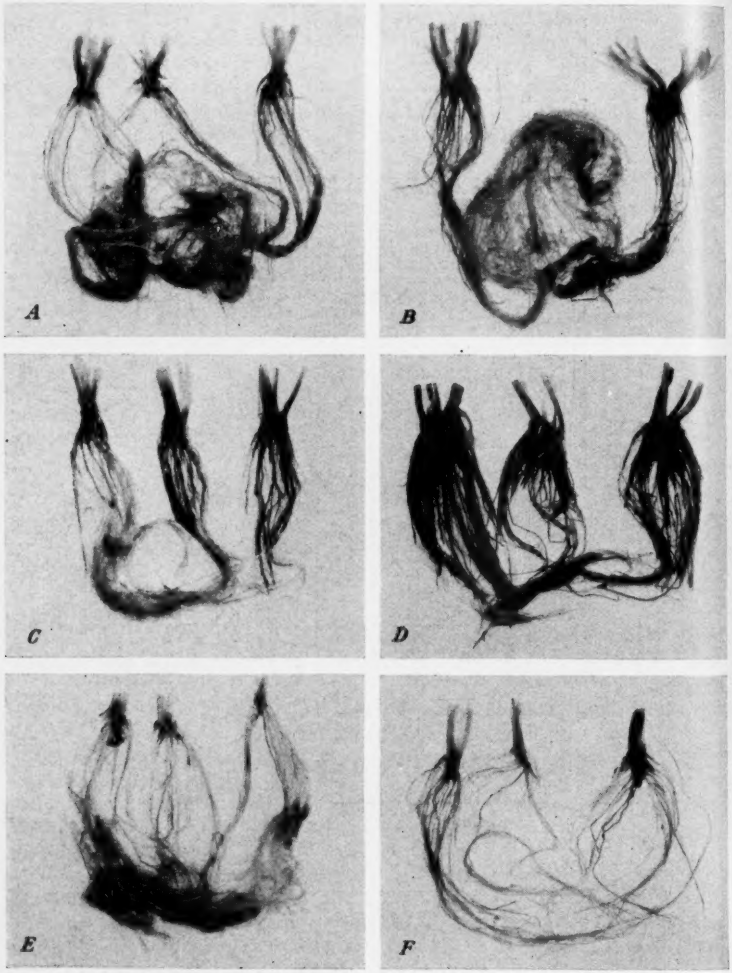

FIGURE 1.—Take-all-diseased wheat roots from untreated soil and from soil that received either phosphorus or nitrogen singly: *A* and *B*, Root systems from untreated soil; *C*, root systems from soil given 1,000 p. p. m. of treble superphosphate; *D*, root systems from soil given a total of 976 p. p. m. of nitrogen; *E* and *F*, root systems from soil given 50 p. p. m. of treble superphosphate. ×0.4.

fig. 2). That excessively high available-phosphorus and nitrate-nitrogen levels were not required for successful control is indicated by the terminal nitrogen and phosphorus values shown in table 4. (See also field levels of available phosphorus given in table 1.) The combination of phosphorus and nitrogen treatments used on soil aliquot 30 permitted development of root systems equal to or superior to those

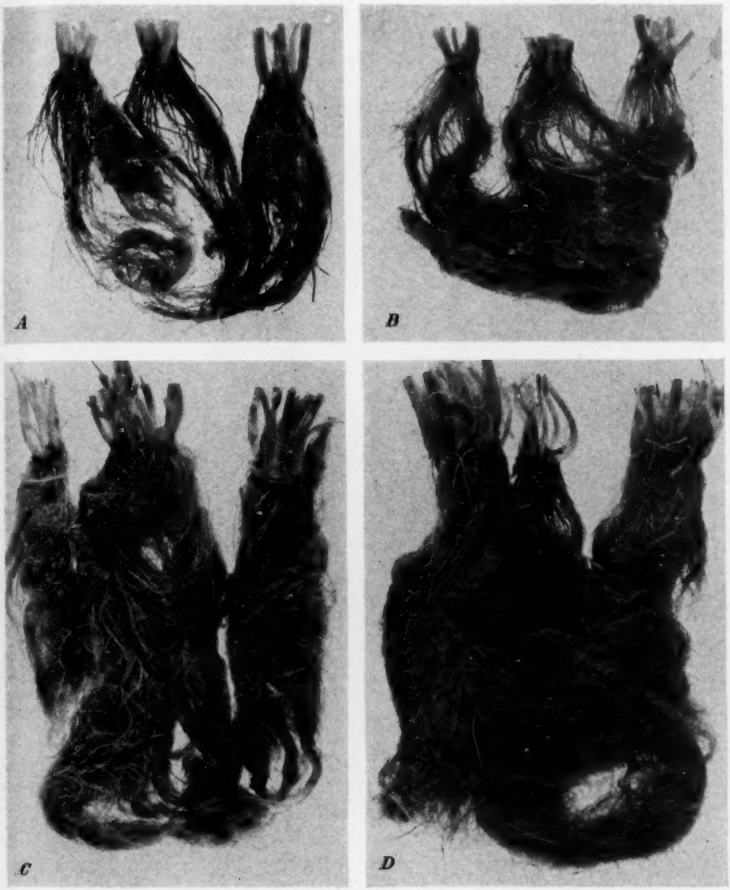

FIGURE 2.—Wheat roots from soil that received both phosphorus and nitrogen amendments: *A* and *B*, Root systems from soil receiving 50 p. p. m. of treble superphosphate and 976 p. p. m. of nitrogen; *C* and *D*, root systems from soil receiving 1,000 p. p. m. of treble superphosphate and 976 p. p. m. of nitrogen. ×0.36.

developed in aliquots of the same infested soil lot fertilized heavily with chopped green alfalfa (fig. 3), a treatment that has been reported to give good control of take-all disease.

EXPERIMENT 3

Each of eight glazed jars received 3,000 gm. of soil from the same lot as used in experiment 2. The following treatments were employed: (1) Check; (2) 200 p. p. m. of phosphorus applied as superphosphate at planting time; (3) 224 mg. of nitrogen per plant, added to the soil as ammonium nitrate in 60 applications of 4 p. p. m. of nitrogen each

during the growing season; and (4) a combination of phosphorus and nitrogen treatments as cited above. All soils were cropped with three plants each. Wheat, vernalized 30 days at 2° C., was grown for 110 days after vernalization; sufficient artificial lighting was employed to provide a daily plant-illumination period in the greenhouse of 16 hours. Upon removal, all plants grown in the untreated or check soil were found to be severely diseased with *Ophiobolus*, as were those grown in soil receiving phosphorus but not nitrogen. With nitrogen fertilization alone, moderate take-all damage was apparent; with both phosphorus and nitrogen supplied, no take-all damage was evident (fig. 4). The results of experiment 2 were considered confirmed.

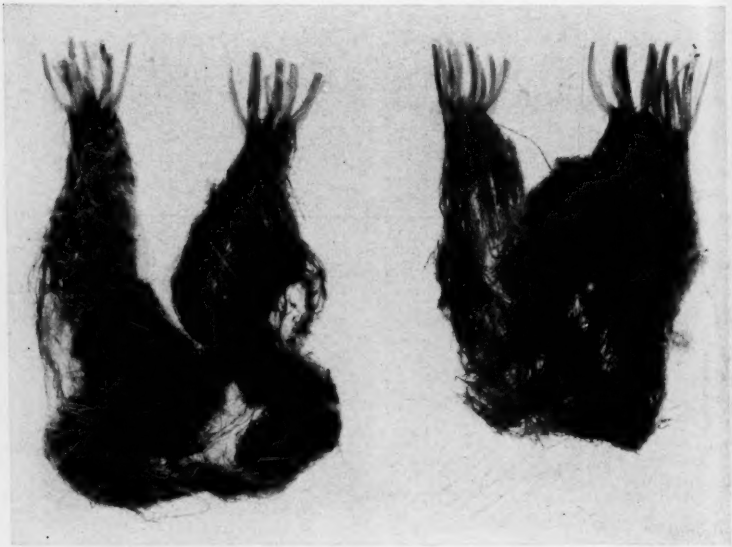

FIGURE 3.—Wheat roots from soil amended heavily with chopped green alfalfa. ×0.36.

DISCUSSION

In the early literature on *Ophiobolus*, unsatisfactory soil-nutrient conditions were frequently considered as predisposing causes of take-all disease (*12*). However, when it was found that inorganic fertilizations failed to give control, especially under greenhouse conditions, host nutrition was assumed to be relatively unimportant, and it was postulated that microbiological antagonism was responsible for the successful control obtained with organic amendments. Although the evidence appears unmistakable that the biological equilibrium of soil does affect the persistence or destructiveness of soil-borne plant pathogens (*6*), it is equally probable that there are other factors involved which at times may limit the incidence or severity of root rot diseases. The present investigations have indicated that for wheat-cropped, *Ophiobolus*-infested soil, nutritional factors are extremely

important in determining the severity of take-all disease. It is believed that the present work is of special value in permitting clarification of some of the apparently contradictory observations concerning take-all disease.

It has been noted above that phosphatic fertilization frequently but not invariably gives field control of take-all, while similar fertilization under greenhouse conditions has generally been found of no value. The available-phosphorus levels reported for eastern-central Kansas soils suggest that there are some fields in which no reduction of take-all could be expected following phosphorus amendments. In such fields, nitrogen is possibly the limiting factor which predisposes the

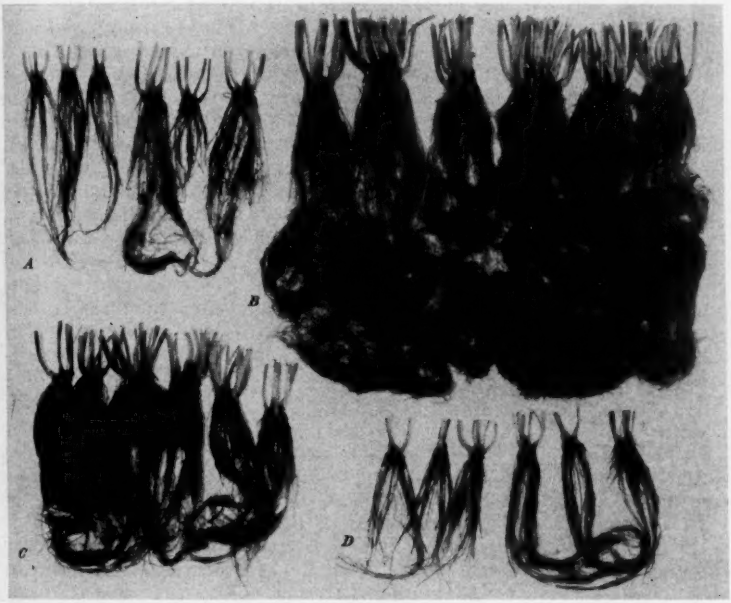

FIGURE 4.—Root systems of wheat plants from experiment 3: *A*, Superphosphate fertilization only; *B*, superphosphate and ammonium nitrate fertilization; *C*, ammonium nitrate fertilization only; *D*, untreated check. ×0.25.

plant to this disease. The frequent failure of phosphatic fertilizations in greenhouse experiments has been shown by the current work to be due to inadequacy of available nitrogen. When the nitrogen content of the soil is maintained at a suitable level, the severity of take-all disease becomes reduced as the available-phosphorus level is raised.

Tillage practices frequently found effective for take-all control may be in part explained by their generally favorable effect upon the accumulation of nitrate nitrogen. That such practices in certain instances fail to provide control may be due to the operation of meteorological or other factors inimical to nitrate-nitrogen accumulation, or to a deficiency of available phosphorus in the soil. Sewell and Call (*15*) have reported upon tillage practices, apparently favorable to accumulation of nitrate nitrogen, which when em-

ployed continuously for several years increased both wheat yield and the severity of take-all disease. It is possible that with the repeated harvesting of heavier yields, the phosphorus deficiency in a soil naturally low in phosphorus might be accentuated to such an extent that the severity of take-all disease would be increased.

The failures reported for inorganic fertilizations under greenhouse conditions are not difficult to understand if one examines both the soil volumes and the rates of fertilizer applications employed. Frequently, only 500 gm. of soil, and occasionally only 200 to 300 gm., per plant have been used (e. g., from 5 to 7 or even 10 plants are reported as grown in unglazed 6-inch pots). This provides only a fraction of the soil volume available to each single wheat plant in the field under normal or optimum stand. That ordinary rates of fertilizer application may not suffice is exemplified by results with potassium nitrate. In field work, 600 pounds per 2,000,000-pound acre is usually considered a heavy application. This same rate (300 p. p. m.) applied to 500 gm. of soil in the greenhouse provides about 18 mg. of nitrogen, or roughly 6 percent of the calculated amount required to produce an average mature wheat plant under field conditions. If all the nitrogen applied at this 300 p.p.m. rate under the greenhouse conditions specified were utilized in plant growth, the response probably could not be detected even if glazed jars were employed. With unglazed pots, losses could be expected because of diffusion of available nitrogen into and from the walls and the bottom openings of such containers. Failure to consider such factors possibly accounts for the fact that control of take-all by means of inorganic fertilizations has not heretofore been reported for greenhouse experiments.

SUMMARY

For wheat-cropped soils of similar texture, differences were not detected in numbers or kinds of micro-organisms in take-all-infested and noninfested field areas, nor was correlation between levels of available phosphorus and incidence of disease apparent.

In greenhouse experiments in which naturally infested soil was given ground wheat straw and inorganic fertilizations at light to heavy field rates at time of planting, the subsequent observations revealed (1) significantly greater soil micropopulations for several months following straw fertilization, (2) unsatisfactory available-phosphorus and nitrate-nitrogen content in soil before the end of the growing season, and (3) at maturity all plants badly damaged by *Ophiobolus graminis*.

When superphosphate fertilization was made at seeding time and inorganic nitrogen, slightly in excess of the amount of nitrogen calculated as necessary for plant growth, was applied during the growing season, it was found that the severity of take-all disease was reduced by increasing rates of superphosphate fertilization. With both available-phosphorus and nitrate-nitrogen content maintained at suitable levels, successful control of take-all disease was obtained, even though significant differences in microbial numbers were not encountered.

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FUNGICIDAL PROPERTIES OF 2,4-DIAMINODIPHENYLAMINE AND OTHER SUBSTITUTED DIPHENYLAMINES [1]

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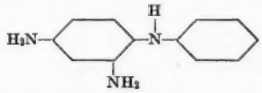
INTRODUCTION

Freshly prepared 2,4-diaminodiphenylamine is a colorless crystalline compound that turns brown upon exposure to air. It is slightly soluble in cold water and gasoline and soluble in alcohol, chloroform, benzene, and morpholine. It has a melting point of 123° C., a flash point of 216° C., and a specific gravity of 1.29. In water at 20° C. it is soluble to the extent of 278 p. p. m. It is prepared by reducing 2,4-dinitrodiphenylamine, which is obtained by reacting 2,4-dinitrochlorobenzene with aniline. It has the following structural formula:



It is used as an antioxidant, an antiplasticizer of unvulcanized rubber, an accelerator of vulcanization, and an activator of other rubber accelerators. It is also used to replace litharge in rubber stocks for insulating wire.

The material is a weak base and is compatible with bentonite-lime mixture, lime, lead arsenate, and calcium arsenate. It is not compatible with nicotine sulfate or rotenone and is inactivated when combined with bentonite and nicotine sulfate-bentonite preparations. It is not easily wetted by water but is easily suspended in water with the help of solvents or wetting agents. It is compatible with sprays containing mineral oils. It is easily handled, without apparent injury to the operator, causes no clogging of spray machinery, and is readily applied when used alone or with adjuvants. Being a dye, it stains the operator's skin and clothes.

In preliminary tests 2,4-diaminodiphenylamine was found to be toxic [2] to several plant pathogens. This paper presents the results of tests to determine the fungicidal properties of this material as well as those of other readily available substituted diphenylamines.

METHODS AND MATERIALS

Since the methods used in the fungicide tests are described fully in previous papers,[3] [4] they need be outlined only briefly here. Conidia

[1] Received for publication September 30, 1941.

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of the peach brown rot organism (*Sclerotinia fructicola* (Wint.) Rehm) were obtained from 7-day-old pure cultures growing at 24° C. on sterilized string beans and conidia of the apple bitter rot organism (*Glomerella cingulata* (Ston.) Spauld. and Von Schrenk) were obtained from 7-day-old pure cultures grown at 24° on oatmeal agar.

The conidia were placed in cubes of water agar and perfused with saturated aqueous solutions of the given chemical for 24 hours. During and after perfusion, sample agar cubes were taken out, the conidia that had germinated were counted, and the degree of toxicity of the solution was recorded. Also, glass cover slips bearing standardized spray residues of the chemical, with or without adjuvants, were suspended in special holders from apple trees so that the residues were subjected to weathering under orchard conditions. At frequent intervals these cover slips were brought into the laboratory and the toxicity of the weathered residues was determined by placing conidial suspensions of the test fungi in contact with the weathered residues inverted over Van Tieghem cells and recording the fungicidal effect on germination.

In addition, limited field tests were conducted by spraying apples with 2,4-diaminodiphenylamine at the rate of 2 pounds to 100 gallons of water, with adjuvants, to control scab, caused by *Venturia inaequalis* (Cke.) Wint., and russet; cherries, to control leaf spot, caused by *Coccomyces hiemalis* Hig.; and peaches, to control brown rot, caused by *Sclerotinia fructicola*, and scab, caused by *Cladosporium carpophilum* Thuem. Also, Red Kidney bean plants (*Phaseolus vulgaris* L.) and small limbs of pear, plum, apricot, and quince were sprayed to determine their tolerance.

As only small quantities of the other diphenylamines were available they were subjected only to laboratory tests and to tests for toxicity on the bean plants.

EXPERIMENTAL RESULTS

FUNGICIDAL ACTIVITY OF 2,4-DIAMINODIPHENYLAMINE

Perfusion and Spray-Residue Tests

Laboratory studies of the fungicidal properties of saturated solutions of 2,4-diaminodiphenylamine are shown in table 1. The saturated solution, prepared by shaking 2 gm. in 2,000 ml. of distilled water, was brown in color, had a strong but not objectionable odor, and contained approximately 278 p. p. m. of the chemical. Its toxicity (table 1) was compared with that of a saturated solution of diphenylamine, which contained 11 p. p. m., and with that of a solution of copper sulfate, which contained 0.50 p. p. m. of Cu, a concentration often present in water in contact with residues of alkaline bordeaux mixtures.

The saturated solution of 2,4-diaminodiphenylamine was extremely toxic to the conidia of *Sclerotinia fructicola* and *Glomerella cingulata* under these conditions, and that of diphenylamine was more toxic than that of copper sulfate.

Table 2 contains data on the toxicity of residues of 2,4-diaminodiphenylamine, combined with various adjuvants, to the conidia of *Sclerotinia fructicola* and *Glomerella cingulata*, as compared with the toxicity of spray residues of bordeaux mixture under the same con-

ditions of orchard weathering. It also shows that 2,4-diaminodiphenylamine residues were toxic and weathered satisfactorily when used with a wetting agent alone or a wetting agent combined with lime, lime and bentonite, mineral oil, and lime and mineral oil; but not when combined with nicotine sulfate and bentonite. In other tests, not shown in the tables, performance was satisfactory when this chemical was used with lead arsenate; but with bentonite alone inactivation occurred. 2,4-diaminodiphenylamine, when present in toxic quantities, stains the conidia of *Sclerotinia fructicola* and *Glomerella cingulata* a deep brown. This staining of the conidia facilitates observations on toxicity and also is an indicator of the amount of the chemical present in the residue. When the chemical is combined with bentonite or with nicotine sulfate and bentonite, its typical color is seen only on the particles of floc formed by the physical contact of the materials, and the union appears so tight that none of the chemical is absorbed by the conidia. This appears not to occur when materials such as lime, lime-bentonite floc, or mineral oil are used with the chemical.

TABLE 1.—*Fungicidal effect of aqueous solutions of 2,4-diaminodiphenylamine, diphenylamine, and copper sulfate on conidia of Sclerotinia fructicola and Glomerella cingulata after various periods of perfusion*

Material			Conidia of *S. fructicola*		Conidia of *G. cingulata*	
Name	Approximate concentration	Period of perfusion	Appearance at end of incubation	Subsequent germination [1]	Appearance at end of incubation	Subsequent germination
	P. p. m.	*Hours*		*Percent*		*Percent*
2,4-diaminodiphenylamine.	278	2	All appeared injured	0	All appeared injured	0
		4	do	0	do	0
		6	do	0	do	0
		22	do	0	do	0
		24	do	0	do	0
Diphenylamine [2]	11	2	Slight injury; no germination.	100	No injury; no germination.	100
		4	Slight injury; few buds	86	do	100
		6	do	22	do	100
		22	All appeared injured	0	All appeared injured	21
		24	do	0	do	0
Copper sulfate (calculated as Cu).	[3] .50	2	No apparent injury	100	No apparent injury	100
		4	do	85	do	100
		6	Slight injury apparent	48	Slight injury apparent	68
		22	All appeared injured	Trace	Many appeared injured.	42
		24	do	Trace	do	50

[1] Transferred to oxidized potato juice 4-percent agar; incubated for 24 hours.
[2] 2 gm. of material added to 2,000 ml. of distilled H_2O.
[3] About the solubility of copper in weathered alkaline bordeaux mixtures.

In these tests of relatively short duration, 2,4-diaminodiphenylamine proved to be effective and comparable with bordeaux mixture in toxicity to the conidia of the two fungi employed. But in none of these tests were the residues subjected to heavy continuous rains, and the data give no clue to what effect extended weathering would have had on toxicity. The high solubility of 2,4-diaminodiphenylamine should be detrimental to its effectiveness under conditions of longer exposure or to excessively heavy precipitation. In some field tests, not recorded in the tables, and conducted where such conditions existed, this was found to be true.

TABLE 2.—*Effect of weathering on the fungicidal activity of residues from 2,4-diaminodiphenylamine plus various adjuvants and from bordeaux mixture sprayed on glass cover slips on conidia of Sclerotinia fructicola and Glomerella cingulata* [1]

Combination	Period weathered in orchard	Precipitation total	Conidia of *S. fructicola* after 24 hours—		Conidia of *G. cingulata* after 24 hours—	
			In Van Tieghem cell	On potato agar [2]	In Van Tieghem cell	On potato agar [2]
	Days	*Inches*				
2,4-diaminodiphenylamine (2 lb.) + hydrated lime (8 lb.) + lead arsenate (2 lb.) + wetting agent [3] (15 gm.) + water (100 gal.)	0		All appeared injured	No germination	All appeared injured	No germination.
	2	0.42	All appeared plasmolyzed	do	All appeared plasmolyzed	Do.
	4	.62	do	do	do	Do.
	7	.62	do	do	do	Do.
	9	.62	do	do	do	Do.
	11	.95	do	do	do	Do.
2,4-diaminodiphenylamine (2 lb.) + hydrated lime (8 lb.) + bentonite (2 lb.) + lead arsenate (2 lb.) + wetting agent [3] (15 gm.) + water (100 gal.)	0		do	do	do	Do.
	2	.48	do	do	do	Do.
	4	.48	do	do	do	Do.
	7	.48	do	do	do	Do.
	9	1.06	do	do	Many appeared normal	Many germinated.
	11	1.06	do	do	do	Moderate germination.
2,4-diaminodiphenylamine (2 lb.) + bentonite (5 lb.) + nicotine sulfate (1 pt.) + water (100 gal.)	0		No apparent injury	Many germinated	46 percent germinated	Many germinated.
	2	.48	do	do	24 percent germinated	Do.
	4	.48	do	do	All germinated	Do.
	7	.48	do	do	do	Do.
	9	1.06	do	do	do	Do.
	11	1.06	do	do	do	Do.
Copper sulfate (8 lb.) + hydrated lime (8 lb.) + lead arsenate (2 lb.) + water (100 gal.)	0		All appeared injured	No germination	All appeared injured	No germination.
	2	.42	All appeared plasmolyzed	do	All appeared plasmolyzed	Do.
	4	.62	Some appeared injured; some appeared not injured.	Moderate germination	No apparent injury	Many germinated.
	7	.62	All appeared plasmolyzed	No germination	Few appeared injured	Moderate germination.
	9	.62			12 percent with buds	Many germinated.
	11	.95	All appeared plasmolyzed	No germination	Few budded	Do.
2,4-diaminodiphenylamine (2 lb.) + wetting agent [3] (15 gm.) + water (100 gal.)	0		do	do	All appeared injured	No germination.
	2	0	do	do	do	Do.
	4	0	do	do	do	Do.
	7	0	do	do	do	Do.
	9	0	do	do	do	Do.
	11	2.28	do	do	do	Do.
2,4-diaminodiphenylamine (2 lb.) + mineral oil (1 qt.) + wetting agent [3] (15 gm.) + water (100 gal.)	0		do	do	All appeared plasmolyzed	Do.
	2	.18	do	do	do	Do.
	4	.18	do	do	do	Do.
	6	.18	do	do	do	Do.
	8	1.18	do	do	do	Do.
	10	1.33	do	do	do	Do.

2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +wetting agent (15 gm.) +water (100 gal.)	0		do	do	do	Do.
	2	0	do	do	do	Do.
	4	Trace	do	do	do	Do.
	6	Trace	do	do	do	Do.
	8	Trace	do	do	do	Do.
	10	.46	do	do	do	Do.
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +mineral oil (1 qt.) +wetting agent [2] (15 gm.) +water (100 gal.)	0		do	do	do	Do.
	1	.93	do	do	do	Do.
	3	.93	do	do	do	Do.
	5	.93	do	do	do	Do.
	7	1.76	do	do	do	Do.
	12	2.18	do	do	do	Do.

[1] All untreated conidia of both species had grown after the 24-hour incubation period in the Van Tieghem cells.
[2] Transferred to potato agar at the end of the 24-hour incubation period in the Van Tieghem cells. (See table 1.)
Santomerse (butylated phenylphenol sodium sulfonate).

Field Tests

Tables 3, 4, and 5 show data from limited field tests of 2,4-diaminodiphenylamine on apple and peach, conducted during the 1939 and 1940 seasons in young orchards on the grounds of the United States Horticultural Station, Beltsville, Md. The 1939 experiments on apple, as shown in table 3, were more reliable than those conducted during 1940, because of the greater number of apples examined. In 1939 scab was well controlled on all varieties and russet was very light, even on the fairly sensitive York Imperial variety. In the 1940 tests the crop was light, but in all cases a considerable reduction of fruit and leaf scab (tables 3 and 4) was effected by the use of 2,4-diaminodiphenylamine combined with lime or with lime and oil. In 1940 russeting was serious even on nonsprayed trees of sensitive varieties, owing to the cold spring. Under these conditions, 2,4-diaminodiphenylamine compared favorably with but was not better than the standard treatments.

Table 3.—*Effect of 6 applications of 2,4-diaminodiphenylamine spray combinations on scab and russet on fruits of various apple varieties*

Year and combination	Variety	Fruits examined	Fruits russeted	Fruits scabbed
1939		*Number*	*Percent*	*Percent*
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +bentonite (2 lb.) +lead arsenate (2 lb.) +wetting agent [2] (15 gm.) +water (100 gal.)	Starking	228	1.3	3.5
	Rome Beauty	645	1.9	Trace
	York Imperial	75	[1] 26.6	0
Untreated	Starking	374	0	83.0
1940				
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +lead arsenate (2 lb.) +mineral oil (1 qt.) +wetting agent [2] (15 gm.) +water (100 gal.)	Delicious	9	0	11.0
	Stayman Winesap	6	0	0
	Grimes Golden	4	100.0	0
	Jonathan	54	22.2	0
	Golden Delicious	11	100.0	0
	York Imperial	43	14.0	2.3
Untreated	Delicious	8	12.5	100.0
	Stayman Winesap	87	2.3	86.2
	Grimes Golden	171	11.6	38.0
	Jonathan	61	80.0	64.0
	Golden Delicious	11	100.0	36.3

[1] Light.
[2] Santomerse (butylated phenylphenol sodium sulfonate).

TABLE 4.—*Effect of 6 applications of 2,4-diaminodiphenylamine spray combinations and of other sprays on scab on leaves of various apple varieties, at harvest, 1940*

[100 leaves examined from each of 3 parts of each tree; "untreated" data based on 4 trees and other data on 1 tree]

Combinaton	Leaves infected on—							
	5-year-old Delicious	4-year-old Delicious	5-year-old Staymman Winesap	4-year-old Staymman Winesap	5-year-old Grimes Golden	5-year-old Jonathan	5-year-old York Imperial	5-year-old Golden Delicious
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +lead arsenate (2 lb.) +wetting agent [1] (15 gm.) +water (100 gal.)	47.0	Trace	12.0	Trace	Trace	Trace	Trace	0
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +lead arsenate (2 lb.) +wetting agent [1] (15 gm.) +mineral oil (1 qt.) +water (100 gal.)	5.0	Trace	Trace	Trace	Trace	Trace	0	0
Carbon sulfur (8 lb.) +lead arsenate (2 lb.) +lime (8 lb.) +water (100 gal.)	25.0	Trace	Trace	Trace	Trace	Trace	Trace	0
Lime and sulfur combinations [2]	Trace	Trace	Trace	Trace	Trace	Trace	Trace	0
Untreated	89.5	75.5	82.6	67.0	72.0	42.0	44.8	32.0

[1] Santomerse (butylated phenylphenol sodium sulfonate).
[2] Liquid lime-sulfur 2–100—pre-pink; lime-sulfur plus lead arsenate 2–2–100—pink and calyx; wettable sulfur, lime, and lead arsenate 8–4–2–100—3 cover applications.

TABLE 5.—*Effect of 5 applications of 2,4-diaminodiphenylamine spray combinations on scab and brown rot of peach fruit*

Year and combination	Variety	Trees	Fruits examined	Scab		Fruit affected by brown rot [2]
				Severity on affected fruit [1]	Fruit affected	
1939		*Number*	*Number*		*Percent*	*Percent*
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +bentonite (2 lb.) +wetting agent [3] (15 gm.) +water (100 gal.)	Alexander	1	108	Trace		37.9
	Carman	1	49	Light	100.0	32.7
	Elberta	1	45	do	100.0	0
Untreated	Alexander	3	430	Moderate	90.0	99.0
	Carman	1	66	Severe	100.0	36.4
	Elberta	6	245	do	100.0	8.1
1940 [4]						
2,4-diaminodiphenylamine (2 lb.) +hydrated lime (8 lb.) +wetting agent [3] (15 gm.) +water (100 gal.)	Alexander	1	258	Light	2.8	1.6
	Carman	1	248	do	5.0	22.6
	Elberta	3	689	do	13.8	6.3
Untreated	Alexander	3	660	Moderate	85.0	42.0
	Carman	3	279	do	71.0	33.3
	Elberta	12	2,510	do	100.0	25.3

[1] Light scab, 1 or 2 spots; moderate scab, several to a dozen spots; severe scab, many spots.
[2] Poor control of insects in check trees, resulting in spread of brown rot.
[3] Santomerse (butylated phenylphenol sodium sulfonate).
[4] Lead arsenate plus zinc-lime added to shuck and first cover sprays to control worms.

In the peach tests, as implied in table 5, the crop of fruit was light in 1939. Curculio and oriental peach moth injury were very serious during both seasons because of the nearness of the treated trees to the unsprayed check trees, and brown rot was more of a factor than usual because these insects produce portals of entry for the brown rot pathogen. In the 1939 tests zinc-lime plus lead arsenate was not added to the 2,4-diaminodiphenylamine sprays, whereas it was added in 1940.

Under the conditions of the peach tests, although the treated trees were flanked on each side with untreated trees, 2,4-diaminodiphenylamine gave a very fine performance against scab and brown rot. Whenever scab was present the lesions were small and scanty, and none of the fruit could be classed as culls because of it. The tests were limited, however, and give only a clue to the behavior of this material against the organisms that cause scab and brown rot under more rigid conditions.

On cherry varieties, tests were conducted with 2,4-diaminodiphenylamine during 1939 and 1940 on Montmorency, Early Richmond, and Saint Medard. In both seasons the material, though protecting against leaf spot when applied frequently, gave no promise of being a protectant over a long period, in which respect it did not compare with bordeaux mixture or with copper phosphate mixture. In both seasons control was good up to the time of the post-harvest application, but leaf spot became severe during the long period following this application, indicating a deficiency in the lasting qualities of the residues of the compound.

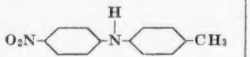
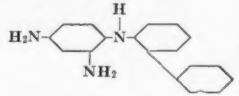
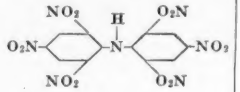
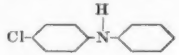
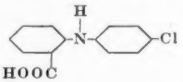
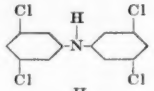
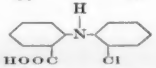
FUNGICIDAL AND PHYTOCIDAL PROPERTIES OF SOME CLOSELY RELATED DIPHENYLAMINES

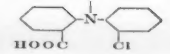
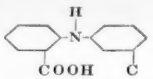
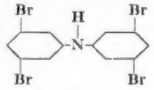
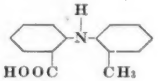
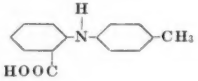
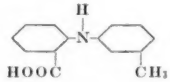
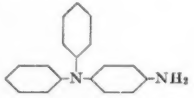
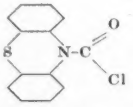
Table 6 shows the relative fungicidal value of 2,4-diaminodiphenylamine and some closely related diphenylamines. Since none of the true isomers of the 2,4-diaminodiphenylamine were available for the tests, the relative value of these will have to await their preparation. Of the closely related compounds, 2′,4′-diamino-2-phenyldiphenylamine and diphenylamine killed the largest percentage of conidia of both *Sclerotinia fructicola* and *Glomerella cingulata*. 4-nitrodiphenylamine, 4-chlorodiphenylamine, and diphenylparaphenylene diamine were toxic to the conidia of *S. fructicola* in varying degrees but not to those of *G. cingulata*. The rest of the compounds exhibited little or no toxicity. It is interesting to note that the 4 position appears to be correlated with toxicity provided no other position is occupied by a dissimilar group. With 2,4-diaminodiphenylamine, both positions are occupied by a similar group.

TABLE 6.—*Fungicidal effect of perfusion in saturated solutions of 2,4-diaminodiphenylamine and of some closely related diphenylamines on the conidia of Sclerotinia fructicola and Glomerella cingulata*

Compound	Structural formula	Solubility in water	Conidia of indicated fungus killed in—									
			2 hours		4 hours		6 hours		22 hours		24 hours	
			Sclerotinia	*Glomerella*	*Sclerotinia*	*Glomerella*	*Sclerotinia*	*Glomerella*	*Sclerotinia*	*Glomerella*	*Sclerotinia*	*Glomerella*
		P. p. m.	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
2,4-diaminodiphenylamine		278	100	100	100	100	100	100	100	100	100	100
Diphenylamine		11	0	0	14	0	72	0	100	79	100	100
Phenothiazine		12	0	0	0	0	0	0	0	0	0	0
2,4-dinitrodiphenylamine		206	0	0	0	0	0	0	0	0	0	0
4-nitrodiphenylamine		Trace	0	0	0	0	0	0	99+	0	100	0
2-nitrodiphenylamine		3	0	0	0	0	0	0	0	0	0	0

TABLE 6.—*Fungicidal effect of perfusion in saturated solutions of 2,4-diaminodiphenylamine and of some closely related diphenylamines on the conidia of Sclerotinia fructicola and Glomerella cingulata*—Continued

Compound	Structural formula	Solubility in water	Conidia of indicated fungus killed in—									
			2 hours		4 hours		6 hours		22 hours		24 hours	
			Sclerotinia	*Glomerella*	*Sclerotinia*	*Glomerella*	*Sclerotinia*	*Glomerella*	*Sclerotinia*	*Glomerella*	*Sclerotinia*	*Glomerella*
4′-nitro-4-methyldiphenylamine		*P. p. m.* 3	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0	*Percent* 0
2′,4′-diamino-2-phenyldiphenylamine		67	39	0	34	0	42	54	74	42	86	48
Dipicrylamine		21	0	0	0	0	0	0	0	0	4	0
4-chlorodiphenylamine		8	0	0	0	0	0	0	49	0	88	0
4′-chlorodiphenylamine-2-carboxylic acid		20	0	0	0	0	0	0	0	0	0	0
3′,5′,3,5-tetrachlorodiphenylamine		14	0	0	0	0	0	0	0	0	0	0
2′-chlorodiphenylamine-2-carboxylic acid		14	0	0	0	0	0	0	0	0	0	0

2'-chlorodiphenylamine-2-carboxylic acid		14	0	0	0	0	0	0	0	0	0	0
3'-chlorodiphenylamine-2-carboxylic acid		6	0	0	0	0	0	0	4	0	12	0
3',5',3,5-tetrabromodiphenylamine		5	0	0	0	0	0	0	0	0	4	0
2'-methyldiphenylamine-2-carboxylic acid		10	0	0	0	0	0	0	0	0	0	0
4'-methyldiphenylamine-2-carboxylic acid		5	0	0	0	0	0	0	0	0	0	0
3'-methyldiphenylamine-2-carboxylic acid		9	0	0	0	0	6	9	14	6	10	6
Diphenylparaphenylene diamine		4	0	0	0	0	0	0	34	0	41	0
Phenothiazine-N-carboxylic acid chloride		13	0	0	0	0	0	0	0	0	0	0

Diphenylamine and 2′,4′-diamino-2-phenyldiphenylamine were the only members of the tested group that were phytocidal. All of these compounds were sprayed on Red Kidney bean plants, and injury was caused only by the two just mentioned. When sprayed on leaves and fruits of peach, cherry, plum, apricot, pear, apple, and quince, 2,4-diaminodiphenylamine in combination with a wetting agent, lime, lead arsenate, lime and bentonite, mineral oil, mineral oil plus lime, and nicotine sulfate plus bentonite caused no injury.

SUMMARY AND CONCLUSIONS

Perfusion, spray-residue, and field tests, conducted during a period of several years, showed that 2,4-diaminodiphenylamine possesses promising fungicidal properties and does not cause injury to apple, pear, peach, plum, cherry, apricot, quince, or bean.

2,4-diaminodiphenylamine is basic in character and is compatible with lime, bentonite plus lime, lead arsenate, and mineral oil. When combined with bentonite or with bentonite flocculated with nicotine sulfate, its toxicity is lost.

2,4-diaminodiphenylamine is difficult to wet in water but is readily dispersed by the addition of a small amount of a wetting agent.

The fungicidal properties of saturated solutions of a series of closely related diphenylamines were also tested. Diphenylamine, one of the parent materials of 2,4-diaminodiphenylamine, and 2′,4′-diamino-2-phenyldiphenylamine approached 2,4-diaminodiphenylamine in toxicity. 4-nitrodiphenylamine, 4-chlorodiphenylamine, and diphenyl-paraphenylene diamine were toxic to the conidia of *Sclerotinia fructicola* but not to those of *Glomerella cingulata*. With the exception of diphenylamine and 2′,4′-diamino-2-phenyldiphenylamine, none of the compounds tested were injurious to Red Kidney bean plants.

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